

Technical University of Denmark



## Effect of frozen storage temperature on quality-related changes in fish muscle

Changes in physical, chemical and biochemical quality indicators during short- and long-term storage

Burgaard, Maria Garver; Jørgensen, Bo Munk

*Publication date:*  
2010

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*

Burgaard, M. G., & Jørgensen, B. (2010). Effect of frozen storage temperature on quality-related changes in fish muscle: Changes in physical, chemical and biochemical quality indicators during short- and long-term storage. Kgs. Lyngby, Denmark: Technical University of Denmark (DTU).

## DTU Library

Technical Information Center of Denmark

---

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

National Food Institute  
Technical University of Denmark  
Mørkhøj Bygade 19  
DK - 2860 Søborg

Tel. +45 35 88 70 00  
Fax +45 35 88 70 01

[www.food.dtu.dk](http://www.food.dtu.dk)

ISBN: 978-87-92158-76-5

# Effect of frozen storage temperature on quality-related changes in fish muscle

Changes in physical, chemical and biochemical quality indicators during short- and long-term storage



Maria Garver Burgaard  
PhD Thesis  
January 2010

# **Effect of frozen storage temperature on quality-related changes in fish muscle**

**Changes in physical, chemical and biochemical quality indicators during short- and long-term storage**

January 2010

Photo:

ISBN: 978-87-92158-76-5

This report is available at

[www.food.dtu.dk](http://www.food.dtu.dk)

National Food Institute  
Technical University of Denmark  
Mørkhøj Bygade 19  
DK-2860 Søborg

Tel: +45 35 88 70 00

Fax: +45 35 88 70 01

## **Preface**

My PhD-project was financed by The Danish Food Industry Agency, subsection of The Ministry of Food, Agriculture and Fisheries. It was done in cooperation with Højmarklaboratoriet A/S, AGA A/S, Norfrig Service A/S and Dairei Europe A/S, all of whom I would like to thank for their collaboration.

Most of all I would like to thank my supervisor Bo Jørgensen for his guidance during this project and for being willing to explain statistical and multi-variate issues over and over again.

I would also like to thank the employees of FF, who makes it a pleasant place to work. In particular, I would like to thank Heidi Olander Petersen, whose help with the analyses, not least during my maternal leave, has been invaluable. I also owe thanks to Inge Holmberg and Ulrik Cold for excellent technical assistance with the analyses and Carsten Østerberg, Jeanette Unger Møller, Hugo Ladefoged and Claus Asperud Reesbøll for practical help. Moreover I would like to thank Jette Nielsen for her interest in my project. I am also very grateful for Søren Tørper Christensen's help with providing the literature used.

Finally, I would like to thank my father-in-law, Steen Garver, for proof-reading of the thesis.

Kgs. Lyngby, January 2010

Maria Garver Burgaard



## Summary

The primary objective of the present work was to investigate the relation between quality-related changes in frozen fish and storage temperatures in the interval from -20 to -80 °C. The design of the storage experiments was paired with regard to temperature and two individuals were stored at each temperature and time. The time dependency was also investigated during one to 18 months of frozen storage, though using different individuals from month to month. Moreover the importance of freezing and thawing rates was investigated in pre-and post-rigor cod.

The following methods were used to describe the changes occurring in the main components (water, protein, lipid) of fish muscle during frozen storage: water content, drip loss, water holding capacity (WHC), nuclear magnetic resonance (NMR) spectroscopy, level of thiobarbituric acid reacting substances (TBARS), carbonyl group determination, colour measurement and activity of the enzymes SR  $\text{Ca}^{2+}$ -ATPase and cathepsin D. Multivariate analysis in combination with 1- and 2-way ANOVA were used to identify changes related to temperature and time.

Ice crystallisation point and amount of unfrozen water in fish muscle during freezing were also investigated using differential scanning calorimetry (DSC). Moreover the mobility of an added spin probe in the unfrozen water fraction of frozen cod muscle was measured using electron spin resonance (ESR).

The main result of the storage experiments is that a storage temperature of -30 °C is sufficiently low for maintaining a high quality of post-rigor cod up to 12 months of frozen storage. In general, storage at -20 compared to -30 °C or lower influenced quality-related changes such as drip loss, WHC,  $\text{Ca}^{2+}$ -ATPase and cathepsin D activity, carbonyl content, colour negatively, whereas the level of TBARS was not influenced. The results of the NMR measurements do, however, indicate that long time storage (more than 12 months) at -40 °C or lower compared to -30 °C or higher results in less pronounced changes in WHC and water distribution and thereby probably also less protein denaturation. Therefore a storage temperature of -40 °C is advisable for storage of cod for more than 12 months if the aim is to preserve the quality in the best way.

The rate of freezing and thawing was important in relation to drip loss and WHC and especially pre-rigor cod was susceptible to handling procedures. In one experiment it was

shown that slow thawing (2 °C cold store) of pre-rigor cod resulted in less drip loss compared to fast thawing (in water), whereas an experiment conducted on high quality pre-rigor cod showed the exact opposite.

For rainbow trout no changes in drip loss, WHC, water distribution and membrane stability ( $\text{Ca}^{2+}$  ATPase and cathepsin D activity) were observed at -20 °C compared to lower temperatures. Storage at -40 °C and lower, did however, reduce the level of secondary lipid oxidation (TBARS) compared to storage at higher temperatures. These results were confirmed in an experiment with five individuals instead of two. The TBARS value for rainbow trout stored for 18 months at -30 °C was below 10  $\mu\text{mol/kg}$  fish, which has been used as a detection limit for rancid taste in mackerel (Ke *et al.*, 1976). Therefore -30 °C seems to be sufficiently low for the prevention of quality deteriorating processes during long term storage of rainbow trout.

Tuna was stored for up to 3 months and in this time interval only changes in secondary lipid oxidation (TBARS) and colour were observed. Storage at -30 °C prevented discoloration (change to brownish colour) and lipid oxidation better than storage at -20 °C.

The results of the DSC experiments showed that super-cooling of more than 10 degrees Celsius occurred during freezing of cod, herring and tuna in a DSC. A relatively large proportion of the muscle water remained unfrozen during freezing down to -80 °C and no significant dependence of temperature on the amount of unfrozen water existed in the interval from -20 to -80 °C. Thus all freezable water was frozen at -20 °C or even higher. The mobility of an added spin probe in the unfrozen water fraction of frozen cod muscle was measured by ESR and decreased in the interval from -9 to -21 °C, indicating ice crystallisation in this interval.

In conclusion, a storage temperature of -40 is recommended for long term storage of cod, whereas -30 °C is sufficiently low for storage of cod for less than 12 months. A temperature of -30 °C is recommended for storage of rainbow trout (up to 18 months) and tuna (up to 3 months).



## Sammendrag (Danish summary)

Hovedformålet med dette arbejde var at undersøge sammenhængen mellem lagringstemperaturer i intervallet fra -20 til -80 °C (10 graders intervaller) og kvalitetsrelaterede ændringer i fisk ved lagring i en til 18 måneder (11 lagringsperioder i alt). Forsøgsdesignet var parret i forhold til temperatur og to individer blev lagret ved hver temperatur og til hver tid. Betydningen af lagringstid blev ligeledes undersøgt, dog med det forbehold at der blev anvendt forskellige fisk fra måned til måned i hovedforsøget, hvilket gav en naturlig variation. Derudover blev vigtigheden af indfrysnings- og optøningshastigheder i præ- og post-rigor torsk undersøgt.

Følgende metoder blev brugt til at beskrive de ændringer, som fandt sted i hovedkomponenterne (vand, protein, fedt) i fiskemusklens under lagringen: vandindhold, dryp tab, vandbindingsevne, kerne magnetisk resonans (NMR) spektroskopi, indhold af thiobarbitursyre reaktive stoffer (TBARS), carbonylgruppebestemmelse, farvemåling og aktivitet af enzymerne SR  $\text{Ca}^{2+}$ -ATPase og cathepsin D. Multivariat dataanalyse suppleret med 1- og 2-vejs variansanalyse (ANOVA) blev brugt til at identificere ændringer relateret til temperatur og tid.

Iskrystallisationspunkt og andel af ufrossent vand i fiskemuskel under indfrysning blev også undersøgt ved brug af differential skanning kalorimetri (DSC). Derudover blev mobiliteten af en tilsat probe i den ufrosne vandfraktion i frossen fiskemuskel målt ved brug af elektron spin resonans (ESR).

Det primære resultat af lagringsforsøgene er, at en lagringstemperatur på -30 °C er tilstrækkelig lav til at bevare en høj kvalitet i post-rigor torsk ved frostlagring i op til 12 måneder. Generelt havde lagring ved -20 °C eller derover en negativ effekt på kvalitetsrelaterede ændringer såsom dryptab, vandbindingsevne,  $\text{Ca}^{2+}$ -ATPase og cathepsin D aktivitet, carbonylgruppeindhold og farve sammenlignet med -30 °C, hvorimod niveauet af TBARS var upåvirket. Resultatet af NMR-målingerne indikerer imidlertid at langtidslagring (mere end 12 måneder) ved -40 °C og derunder resulterer i mindre omfattende ændringer i vandbindingsevne og vandfordeling i musklen og dermed sandsynligvis også i formindsket proteindenaturering sammenlignet med lagring ved -30 °C. En lagringstemperatur på -40 °C er derfor anbefalelsesværdig ved lagring af torsk i mere end 12 måneder, hvis målet er at opretholde kvaliteten bedst muligt.

Indfrysnings- og optøningshastighed havde betydning for dryptab og vandbindingsevne og især præ-rigor torsk er særligt følsom. Resultaterne af forforsøget viste, at langsom optøning af præ-rigor torsk (2 °C kølerum) resulterede i et formindsket dryptab sammenlignet med hurtig optøning (i vand), hvorimod resultaterne af forsøget med højkvalitets præ-rigor torsk viste det modsatte.

I regnbueørred resulterede lagring ved -20 °C ikke i ændringer i kvalitetsrelaterede parametre såsom dryptab, vandbindingsevne, vandfordeling i musklen, farve og membranstabilitet ( $\text{Ca}^{2+}$ -ATPase og cathepsin D aktivitet) sammenlignet med lagring ved lavere temperaturer. Lagring ved -40 °C og derunder resulterede derimod i et lavere niveau af sekundær lipidoxidation (TBARS) i forhold til lagring ved højere temperaturer. Disse resultater blev bekræftet i et forsøg med måling på fem individer frem for to. TBARS værdien for regnbueørred lagret i 18 måneder var under 10  $\mu\text{mol/kg}$  fisk, som er blevet angivet som detektionsgrænse for harsk bismag i makrel (Ke *et al.*, 1976) og lagring ved -30 °C må derfor betragtes som tilstrækkeligt lavt til at forhindre kvalitetsforringende processer i langtidslagret regnbueørred.

Tun blev lagret i tre måneder og i denne periode blev der observeret ændringer i sekundær lipidoxidation (TBARS) og i farve. Lagring ved -30 °C forhindrede misfarvning (ændring til brunlig farve) og lipidoxidation bedre end lagring ved -20 °C.

Resultaterne af et DSC eksperiment viste, at en underafkøling på over 10 grader celsius forekommer ved indfrysning af torsk, sild og tun i et differential skanning kalorimeter. En relativt stor andel af muskelvandet forblev ufrossent ved nedfrysning til -80 °C og der var ingen signifikant sammenhæng mellem andelen af ufrossent vand og temperaturen i intervallet -20 til -80 °C. Dermed er alt frysbart vand frossent ved -20 °C eller endog højere. Mobiliteten af en tilsat spin probe i den ufrosne vandfraktion i frossen torskemuskel blev målt ved brug af ESR og denne faldt i intervallet fra -9 til -21 °C, hvilket indikerer at iskrystallisation fandt sted.

## Abbreviations

ANOVA:	Analysis of variance
ATP:	Adenosine triphosphate
$a_w$ :	Water activity
DMA:	Dimethylamine
DMSO:	Dimethyl sulfoxid
DNPH:	Dinitrophenyl hydrazine
DSC:	Differential scanning Calorimetry
DTA:	Differential thermal analysis
EDTA:	Ethylenediaminetetraacetic acid
ESR:	Electron spin resonance
FRAP:	Fluorescence recovery after photo bleaching
FFA:	Free fatty acids
$\Delta H_s$ :	Specific latent heat of fusion
HQ cod:	Pre-rigor cod regarded as of very high quality used in the HQ cod experiment
$m_s$ :	Sample mass
NIR:	Near infra red
NMR:	Nuclear magnetic resonance
$P_i$	Inorganic phosphate
PC	Principal component
PCA:	Principal component analysis
PLS:	Partial least squares regression
PSF:	Pressure shift freezing
PUFA:	Polyunsaturated fatty acids
PV:	Peroxide value
Reg. cod:	Post-rigor cod used in the HQ cod experiment
SR $Ca^{2+}$ -ATPase:	Sarcoplasmic reticulum $Ca^{2+}$ -ATPase
$T_e$ :	Eutectic point
$T_m$ :	Melting point
TBARS:	Thiobarbituric acid reacting substances
TEMPOL:	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
TMAO:	Trimethylamine oxide
$w_u$ :	Fraction of unfrozen water (ratio between unfrozen and total water content)
$w_f$ :	Water content
WHC	Water holding capacity

## List of papers

The following papers are inserted after the References section and referred to in the text as Paper I-III.

Paper I:

Burgaard and Jørgensen (2010). Effect of temperature on quality-related changes in cod (*Gadus morhua*) during short- and long-term frozen storage. Submitted to *Journal of Aquatic Food Product Technology*.

Paper II:

Burgaard and Jørgensen (2010). Effect of frozen storage temperature on quality-related changes in rainbow trout (*Oncorhynchus mykiss*) and yellowfin tuna (*Thunnus albacares*). Accepted for publication in *Journal of Aquatic Food Product Technology*.

Paper III:

Burgaard and Jørgensen (2010). Ice crystallisation point and unfrozen water in fish muscle. Manuscript.

## Contents

1	Introduction .....	11
2	Fish muscle .....	13
2.1	Composition.....	13
2.2	Structure.....	13
2.3	Muscle contraction and rigor mortis.....	14
3	Water in foods .....	17
3.1	Scientific approach to water in foods .....	17
3.2	Water binding, mobility and distribution.....	18
4	Freezing of fish.....	21
4.1	Ice crystal nucleation and formation.....	21
4.2	Freezing point depression .....	21
4.3	Unfrozen muscle water .....	22
4.4	Ice crystal formation in pre- and post-rigor muscle.....	23
4.5	Time-temperature curves .....	24
5	Quality-related changes in frozen fish muscle .....	27
5.1	Factors affecting protein changes .....	27
5.1.1	Dehydration and the effect of inorganic salts.....	28
5.1.2	Changes in lipids and fatty acids.....	29
5.1.3	Enzymatic breakdown of TMAO .....	30
5.2	Changes in functional properties caused by protein changes .....	31
6	Experimental design, materials and methods .....	33
6.1	Methodological considerations .....	33
6.2	Experimental design .....	33
6.2.1	Pilot experiment .....	35
6.2.2	Main experiment .....	36
6.2.3	Tuna experiment.....	37
6.2.4	High quality cod experiment.....	38
6.2.5	Rainbow trout experiment.....	39
6.2.6	DSC-experiments .....	40

6.2.7	ESR experiment.....	41
6.3	Choice of methods .....	41
6.4	Methods .....	43
6.4.1	Drip loss, water content and water holding capacity .....	43
6.4.2	Low field NMR spin-spin relaxation measurements.....	44
6.4.3	Enzyme activity.....	44
6.4.4	TBARS.....	45
6.4.5	Colour.....	45
6.4.6	Lipid content and fatty acid composition.....	46
6.4.7	Carbonyls .....	46
6.4.8	DSC measurements .....	46
6.4.9	ESR measurements.....	48
6.4.10	Data analysis .....	48
7	Results and discussion.....	51
7.1	Pilot experiment.....	51
7.2	Storage experiments.....	52
7.2.1	Composition .....	53
7.2.2	Effect of temperature on drip loss and water holding capacity.....	54
7.2.3	NMR.....	58
7.2.4	Membrane stability measured as $\text{Ca}^{2+}$ -ATPase and cathepsin D activity.....	60
7.2.5	Secondary lipid oxidation measured as TBARS .....	62
7.2.6	Protein oxidation measured as carbonyl group determination .....	64
7.2.7	Colour.....	65
7.2.8	Overview of reported quality-related changes in frozen fish.....	67
7.3	DSC experiments.....	70
7.4	ESR experiment.....	77
8	Conclusion.....	83
9	References .....	85

## 1 Introduction

The freezing of fish is an effective way of long term preservation and it has been shown that fish stored for up to three months under ideal conditions cannot be distinguished from fresh fish regarding colour, taste and texture (Cappeln *et al.*, 1999; Nielsen and Jessen, 2007).

Freezing and frozen storage of fish muscle may, however, lead to denaturation and aggregation of especially myofibrillar proteins. These changes result in altered functional properties, changed textural attributes and reduced water holding capacity and juiciness. The result is a hard, dry and fibrous fish product with a reduced eating quality. Moreover, liquid losses may also directly result in economic losses (Barroso *et al.*, 1998).

Many studies have investigated the relation between storage temperature, time and quality-related changes in fish muscle, but most of these focuses on the temperature interval down to -30 °C with the use of a single lower reference temperature. Moreover, the experimental designs of most other studies are paired regarding storage time and not temperature, making it more difficult to distinguish effects of temperature from individual variation. Therefore little is known about the relation between loss of quality, storage time and storage temperature in the interval between -30 and -80 °C. Also, little is known about to which degree different quality deteriorating processes occur at different temperatures.

The main focus of this study is the effect of various temperatures on quality-related changes in the fish muscle during frozen storage as well as explaining which physical and biochemical processes lead to loss of quality during frozen storage of fish. Therefore it was important to store the fish at as many temperatures as possible and using a variety of analytical analyses to cover different aspects of quality deterioration. It was also of high importance to use a paired experiment with regard to temperature and thereby be able to analyse the same individual after storage at different temperatures. The drawback of this design was, however, that for practical reasons it was only possible to use two replicates at each temperature and it was necessary to use different individuals from month to month.

An experimental design was set up with eight different storage temperatures from -10 to -80 °C (10 °C intervals) and 11 storage periods (1, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 18 months respectively). Cod and rainbow trout were used as model fish representing lean and fatty fish species. Quality-related changes during frozen storage was measured using the following analyses: water content, drip loss, water holding capacity (WHC), nuclear magnetic

resonance (NMR) spectroscopy, level of thiobarbituric acid reactive substances (TBARS), carbonyl group determination, colour measurement and activity of the enzymes SR  $\text{Ca}^{2+}$ -ATPase and cathepsin D.

These different analyses should provide information about the degree of protein oxidation, protein denaturation, lipid oxidation, membrane stability (enzyme activities) and changes in the water holding capacity and water distribution in the fish muscle during storage. The results were analysed using uni- and multivariate data analyses after each storage period giving the opportunity to change the experimental design if large differences between storage temperatures below  $-30\text{ }^{\circ}\text{C}$  appeared early in the storage experiment.

To confirm or investigate some of the results of the main experiment described above further, some experiments with more individuals, other species and with pre- as well as post-rigor fish were also carried out. Moreover, ice crystallisation temperature, amount of unfrozen water at different temperatures and mobility of an added spin probe in the unfrozen water fraction of frozen fish muscle were determined in some additional experiments.



## 2 Fish muscle

In this chapter the composition of fish is shortly described followed by a brief survey of the structure and composition of fish muscle.

### 2.1 Composition

Fish is composed of approximately 20 % protein and 80 % water and lipid.

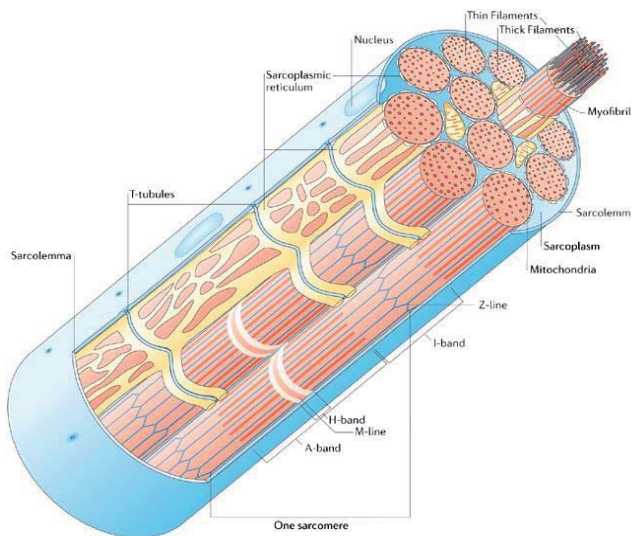
The lipid content varies from approximately 0.2 to 25 % and is divided into two main groups: phospholipids and triglycerides. The phospholipids are also called the structural lipids because they make up the structure of the cell membranes. In lean fish species with lipid contents of less than 1 %, about 90 % are phospholipids. Triglycerides are used as an energy source and are deposited in fat cells distributed in the whole body, but primarily located in the belly flap, in the muscles, that move the fins and tail, in the liver and also in the abdominal cavity. The division of fish into lean and fatty species is based on the way the different species deposit their lipids. Lean species deposit lipid in the liver and fatty species in the fat cells. Fish have a high content of polyunsaturated fatty acids (PUFA) and are therefore susceptible to lipid oxidation. During frozen storage, lipid oxidation occurs in lean as well as fatty species. Oxidation of the phospholipids in lean species results in cold-store flavour and oxidation of triglycerides in more fatty species results in a rancid taste and odour.

The proteins in fish muscle are divided into three groups: the myofibrillar proteins accounting for 70-80 %, the sarcoplasmic proteins accounting for 25-30 % and the stroma proteins (collagen) accounting for 3 % in bony fish and 10 % in cartilaginous fish. The denaturation and aggregation of especially the myofibrillar proteins during frozen storage lead to textural changes such as loss of juiciness and a hard and fibrous product (Sikorski *et al.*, 1976; Shenouda, 1980; Careche *et al.*, 1998; Careche *et al.*, 1999). The quality-related changes in lipids and proteins during frozen storage are further described in chapter 5 and the role of water in chapter 3.

### 2.2 Structure

Fish muscle is composed of striated muscle fibres arranged in a parallel, longitudinal fashion. The muscle fibres are separated by connective tissue, which divides the muscle fibres into 'W'-formed segments called myotomes. A myotom is composed of fibres arranged parallel to

each other and is only one cell long. The ends of the cell or fibre are attached to the sheets of connective tissue, also called the myocommata. Each muscle cell contains sarcoplasm, nucleus, glycogen granules, mitochondria and a number of myofibrils, which run from one end of the fibre to the other. The myofibrils are primarily composed of the thin and thick contractile protein filaments actin and myosin, which are arranged in a way that gives the muscle its striated look (in a microscope) and divides the myofibrils into segments called sarcomeres as illustrated in Figure 1. Myosin accounts for about 40-60 % of the myofibrillar protein content and actin for 15-30 %. Myosin is more sensitive towards frozen storage than actin as described in chapter 5 (Mackie, 1993; Badii and Howell, 2002a).



**Figure 1. Illustration of muscle composition.** Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, copyright: Davies and Nowak (2006).

### 2.3 Muscle contraction and rigor mortis

Each myofibril is surrounded by the sarcoplasmic reticulum, which contains very high concentrations of  $\text{Ca}^{2+}$ . In brief, when the sarcoplasmic reticulum receives a signal from the nervous system, it releases  $\text{Ca}^{2+}$  to the sarcoplasm resulting in the binding of the myosin head to the actin filament. Actin then slides in between the myosin filaments with the use of energy released from the dephosphorylation of ATP, and consequently the muscle contracts. When the nerve signal stops, the concentration of  $\text{Ca}^{2+}$  in the cytosol falls again, the myosin head is released from the actin filament and the contraction ends. In post-mortem muscle, the energy

stores are depleted, ATP cannot be regenerated and the sarcoplasmic reticulum is no longer able to maintain a low level of  $\text{Ca}^{2+}$  in the cytosol. This results in the binding of myosin to actin and thereby the development of rigor mortis (Mackie, 1993). The time after death before onset of rigor mortis is important in relation to the handling (filleting, freezing) of the fish. Differences in the ice formation during the freezing of pre- and post-rigor fish are described in section 4.4.



### 3 Water in foods

#### 3.1 Scientific approach to water in foods

Water is the main constituent of most foods. It accounts for 65-80 % of fish muscle and is solvent for different salts, enzymes and other non-fat food solids. It also functions as a plasticizer of non-crystalline food components (Slade and Levine, 1995). The amount of water and its physical state in foods are important properties, influencing quality, shelf life and processing. At present there are three main approaches to the study of water relations in food systems: water activity ( $a_w$ ), food polymer science and molecular water mobility (Schmidt, 2007).

Water activity can be used to predict the growth of food spoilage organisms (Scott, 1953; Christian and Scott, 1953) and many chemical and biological reactions are a function of  $a_w$ , but only under equilibrium (Labuza *et al.*, 1970). As most foods are non-equilibrium systems, several researchers have questioned the meaningfulness of the use of  $a_w$  in relation to stability of foods (Franks, 1982; Slade and Levine, 1991; Van der Berg, 1991). Slade and Levine (1991) were the first to introduce the glass transition concept to the study of water relations in foods and called it the food polymer science. The third approach is the measurement of molecular water mobility in foods, typically by the use of NMR spectroscopy and imaging, alternatively by measuring the microviscosity using electron spin resonance (ESR), fluorescence recovery after photo bleaching (FRAP) or radiation tracking (Schmidt, 2007; Isengard *et al.*, 2008).

Water mobility can be categorised into different groups according to the type of molecular motion: vibrational, rotational and translational. Vibrational motion is intra-molecular and changes the shape of the molecule, but not the location. Rotational motion is the in-place spinning of a whole molecule, resulting in a change of orientation. Translational motion is the change in location of a whole molecule caused by diffusion or flow. The measurement of  $a_w$  is based on translational mobility of water molecules. If food is placed in a little closed chamber, water from the inside of the food will diffuse out into the chamber due to differences in the chemical potential. This results in a partial vapour pressure of water, which can be measured and used to determine  $a_w$ . In the study of glass transitions different kinds of water mobility are measured depending on the method used. NMR and differential scanning calorimetry (DSC) measurements can detect rotational and translational mobility,

respectively. The measurement of molecular water mobility by the use of relaxation NMR detects rotational mobility of the liquid phase of water. In ESR, FRAP and radiation tracking the rotational and translational mobility of a small probe molecule is measured and the mobility or viscosity of water determined from the tracer's diffusion (Schmidt, 2007; Isengard *et al.*, 2008).

The three different approaches are not necessarily competitive but can complement each other as the common underlying principle is the study of mobility of water in relation to food stability, though on different time and distance scales. The choice of approach, therefore depends on the specific information needed about the system and the use of the data obtained (Schmidt, 2007).

In the present study the focus is on the mobility and distribution of muscle water, measured by relaxation NMR, as affected by storage temperature and time. Water mobility in frozen cod as a function of temperature is measured using ESR spectroscopy and the fraction of unfrozen water in fish muscle at different temperatures is measured using DSC. DSC is also used to determine the ice crystallisation temperature in cod, tuna and herring. The ability of the thawed muscle to hold its own cellular fluid when centrifuged was also measured traditionally as water holding capacity.

### **3.2 Water binding, mobility and distribution**

Water in muscle is distributed throughout the tissue with approximately 90 % located intra-cellular and 10 % extra-cellular (Schnepf, 1989). The water is physically separated by cellular structures such as membranes, but as these are water permeable, the intra- and extra-cellular water can exchange, for example due to changes in osmotic pressure. Intra-cellular water or muscle water is thought to be distributed between different states or populations of water ('pools') characterised by differences in water mobility due to different degrees of binding or association to proteins (Ruan and Chen, 1998). A small portion of the water molecules (less than 0.3 g water/100 g protein) are structurally bound to proteins and show a very different behaviour than that of bulk water (Schnepf, 1989; Isengard *et al.*, 2008). The main fraction of water interacts with proteins to different degrees and the physical retention of this water is dominated by the association with the myofibrillar structure (Schnepf, 1989)

As described above the rotational mobility of water molecules is typically measured by relaxation NMR. Based on the relaxation curves, the number and size of different water pools

can be determined by the use of different two- or three-way chemometric methods. Changes in the water distribution of a certain sample can indicate quality-related changes due to for example storage temperature or time (Jepsen *et al.*, 1999; Jensen *et al.*, 2002).

In porcine meat, three different water pools have been identified, the fastest relaxing reflecting water tightly associated with macromolecules, the intermediate reflecting water located within highly organized protein structures and the slowest relaxing reflecting the extra-myofibrillar water containing the sarcoplasmic protein fraction (Bertram *et al.*, 2001). In fresh and pre-frozen cod, respectively, two to three and three (intact, minced and centrifuged cod) and four (minced cod) different water pools have been identified, the number depending on storage conditions (Andersen and Rinnan, 2002; Jensen *et al.*, 2002; Andersen and Jørgensen, 2004).





## 4 Freezing of fish

When a food is frozen the temperature is reduced to the freezing point by the removal of sensible heat. As ice crystals are formed the latent heat of crystallisation is removed as well as that of other components. Water has a high specific heat capacity ( $c = 4.19 \text{ J/g}\cdot\text{K}$ ) and a high latent heat of fusion ( $\Delta H_s = 334 \text{ J/g}$ ) and the major amount of energy needed to freeze muscle is due to water. In this chapter the process of freezing is described in terms of ice crystal formation in pre- and post-rigor muscle, freezing point depression and importance of freezing rate. The significance of an unfrozen water fraction in frozen muscle is also described.

### 4.1 Ice crystal nucleation and formation

The formation of ice crystals is proceeded by nucleation, which can be homo- or heterogeneous. Supercooling is the driving force for ice nucleation and is defined as the difference between the actual temperature and that of the solid-liquid equilibrium. In a supercooled liquid, homogenous nucleation only occurs if the diffusing molecules spontaneously form a nucleus with a similar structure as ice and with a critical size making it energetically favourable for other water molecules to join. In foods, heterogeneous nucleation is most likely to occur, as a nucleus can form around suspended particles or a cell wall during supercooling. The number of nuclei formed in homo- as well as heterogeneous nucleation increases with increasing degree of supercooling and is crucial for the number and size of ice crystals formed. Apart from the degree of supercooling, the probability of nucleation also depends on the size or volume of the samples because of the statistical nature of the process (Love, 1970; Martino *et al.*, 1998; Wolfe and Bryant, 2001).

### 4.2 Freezing point depression

The freezing point of food is a critical factor for the determination of many physical properties such as freezing time (Planck's equation), water activity, water distribution, amount of frozen water and thawing time (Rahman and Driscoll, 1994).

In fish muscle the freezing point is depressed below that of pure water because of small solutes present in the muscle water. The extent of this depression is approximately proportional to the osmotic pressure of the solution and results in a freezing point depression of about one degree Celsius in bulk muscle water (Ross, 1978; Roos, 1986; Wolfe *et al.*,

2002). The freezing point is often referred to as ‘the equilibrium freezing point’, and can be defined as the temperature at which a minute ice crystal is about to dissolve in melting (Sei and Gonda, 2006). Others use the term ‘initial freezing point’ (James *et al.*, 2005), which is the temperature at which ice crystallisation begins. The ice crystallisation temperature is always below the equilibrium freezing point because supercooling is the driving force for nucleation and ice crystallisation. As described in 4.5 ice crystallisation is followed by the release of latent heat resulting in a rise in temperature to the equilibrium freezing point (Rahman and Driscoll, 1994; Fernandez *et al.*, 2008). The equilibrium freezing point is often estimated from DSC thermograms using either the inflexion point at the left part of the endothermic melting peak (Sablani *et al.*, 2007) or the so-called ‘onset temperature’ which is the intercept between the tangent at this inflexion point and the baseline. The cooling/freezing curve method is also used to determine the equilibrium freezing point (Rahman, 1995; Kasapis *et al.*, 2000; Sablani *et al.*, 2004). Reported equilibrium freezing points of fish muscle and seafood are: -0.68 °C for king fish (Sablani *et al.*, 2007), -1.4 °C for tuna (Rahman *et al.*, 2003), -0.9 °C for abalone (Sablani *et al.*, 2004) values between -0.5 and -2.1 °C for squid, calamari, scallop, cuttle, mussel, octopus, and king prawn (Rahman and Driscoll, 1994), -0.83, -0.91, -0.83 °C for haddock, cod and sea perch respectively (Fikiin, 1998) and -5 °C for tuna (Agustini *et al.*, 2001).

### **4.3 Unfrozen muscle water**

Freezing is an effective way of preservation because the crystallisation of water results in a more concentrated solution and thus a lowering of the water activity in the food. Water associated with macromolecules, membranes and other ultra structural elements in cells and tissues can, however, remain unfrozen at tens of degrees Celsius below the equilibrium freezing point of a bulk solution. Even in the presence of ice crystals, this water remains unfrozen due to a combination of the hydration effect, the presence of small solutes, very high viscosity and small dimensions between membranes and macromolecules (Wolfe *et al.*, 2002). It is therefore frequently named ‘unfreezable’ water, but in compliance with the recommendations of Franks (1986) and Wolfe *et al.* (2002), it is referred to as ‘unfrozen water’ throughout this thesis. The existence of an unfrozen water fraction in frozen foods is the main reason why chemical quality deteriorating processes occur in frozen foods. Several experimental values for the amount of unfrozen water in different muscle foods are reported

in the literature: 9.5 % in cod muscle at -40 °C (Riedel, 1956), 11.0 % in haddock muscle at -40 °C (Charm and Moody, 1966), 15.1 % in reindeer meat at -80 °C (Roos, 1986), 26.8 % in cod at -20 and -60 °C (Paper III), 31.0 % in fresh ground beef meat at -40 °C (Aktas *et al.*, 1997a) and 36.7 % in king fish at -90 °C (Sablani *et al.*, 2007). The relatively big differences between the reported values are probably due to differences between species and methodological differences.

#### **4.4 Ice crystal formation in pre- and post-rigor muscle**

As described in section 3.2, water in fish muscle is distributed among different pools, defined by the mobility of the water which depends on how tight the water is associated with proteins, macromolecules and membranes. Intra- and extra-cellular water and water located in different pools differ in concentration of different solutes and therefore have slightly different freezing points. Whether ice crystals begin to appear intra- or extra-cellularly depends on factors such as the state of the fish muscle (i.e. pre-, in- or post-rigor muscle), cooling rate, concentration of solutes and number of ‘motes’, which can act as nuclei for the ice crystals (Rahman, 2001).

The slow cooling of post-rigor muscle typically results in the formation of extra-cellular ice crystals that advance into the spaces between the unfrozen cells. As ice crystals are formed, the extra-cellular solution, which contains more than 1 % mineral substances, becomes more concentrated. The increased extra-cellular osmotic pressure causes water to leave the cells, thereby depressing the intra-cellular freezing point. The water, which diffuses out of the cells, freezes and contributes to the advancing ice mass. When the ice front has passed the cells, they can be so dehydrated that they, despite the low temperature, do not freeze at all due to high concentrations of solutes and a very high viscosity (Love, 1970; Bevilacqua *et al.*, 1979; Wolfe and Bryant, 2001).

Faster freezing of post-rigor muscle also results in the initial formation of extra-cellular ice. The formation of extra-cellular ice still dehydrates the cells to some extent, but as the temperature decreases rapidly, the cells become supercooled and the remaining intra-cellular water freezes before it has time to diffuse out of the cell. The size and number of intra-cellular ice crystals, respectively decreases and increases as the freezing rate increases resulting in many small spear- or needle-formed discontinuous ice crystals in rapidly frozen muscle (Love, 1970; Bevilacqua *et al.*, 1979).

In pre-rigor muscle, the cell fluids are associated to intra-cellular proteins and the diffusivity from inside to outside the cell is therefore limited resulting in the formation of the intra-cellular ice crystals independent of freezing rate. If very slow freezing rates are used the muscle can however, go into rigor mortis during the freezing process, and ice crystal formation will then be extra-cellular (Love and Haraldsson, 1961; Love, 1970).

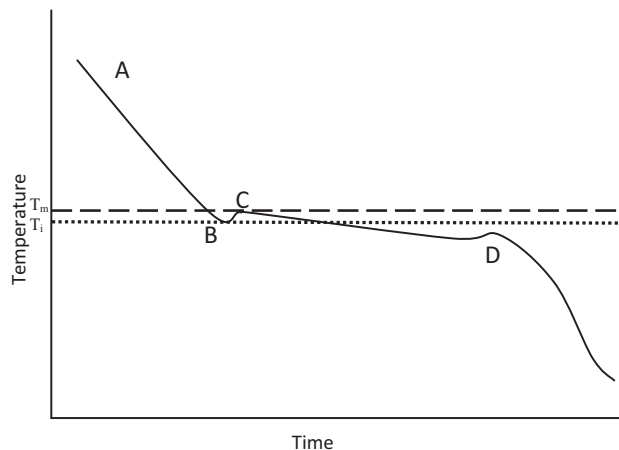
If very high cooling rates are used in combination with cryoprotectants the viscosity of the intra- and extra-cellular solution rises sharply and can become so high that molecular diffusion is halted and the probability of nucleation becomes negligible. This process is called vitrification and the resulting glass is by definition in a state of stable non-equilibrium (Wolfe and Bryant, 2001).

Regardless of initial freezing rate, changes in the size and distribution of ice crystals occur during frozen storage. Re-crystallisation typically takes place during transport or storage due to fluctuations in temperature. An increase in temperature causes small crystals to melt while the following decrease in temperature causes larger crystals to grow. Re-crystallisation includes a number of phenomena such as changes in the number, size, shape, orientation or perfection of the ice crystals, all resulting in a decreased surface-to-volume ratio, which is energetically more favourable (Fennema, 1973; Fernandez *et al.*, 2008). In a study by Chen and Pan (1997) decreased freezing rate of tilapia muscle resulted in increased extra-cellular spacing owing to larger ice crystals. The extra-cellular spacing increased further during storage for 1-2 months at -20 compared to -40 °C. Storage for 6 months resulted, however, in severe fragmentation of muscle fibre bundles at both temperatures. A similar result was obtained by Alizadeh *et al.* (2007) who concluded that higher freezing rates (or increased pressure in pressure shift freezing (PSF)) resulted in an increased number of small ice crystals in salmon samples. These ice crystals were stable during the first 3 months of frozen storage at -20 °C, but increased in size after 6 months. Larger ice crystals obtained using PSF with a lower pressure or by air blast freezing, were stable in size.

#### **4.5 Time-temperature curves**

During the freezing of food a characteristic time-temperature curve is obtained (see Figure 2). From A to B heat is removed and while the temperature of the food decreases to B, water remains liquid due to supercooling. At B the critical number of nuclei is reached and the crystallisation process begins. Latent heat of fusion is liberated faster than heat is being

removed from the system and therefore the temperature rises to C, which is the equilibrium freezing point. From C to D heat is removed from the food at the same rate as before, crystallisation of water continues and latent heat of crystallisation is released. The temperature remains almost constant but decreases a little as the freezing point is depressed by increased solute concentration. In solutions crystals may form and the release of latent heat of solute crystallisation causes the temperature to rise to the eutectic point, D, of the solute. In foods, these eutectic points are difficult to determine, because many solutes are present in small quantities giving only rise to minor increases in temperature. As the temperature drops further, the unfrozen solution becomes more and more concentrated until all ‘freezable’ water has crystallised, and the temperature of the food drops to that of the freezer. Even at very low temperatures a proportion of the water remains unfrozen as described in the previous section (Love, 1970; Rahman, 2001).



**Figure 2.** Time-temperature curve for the freezing of food.  $T_m$ : equilibrium freezing point/melting point,  $T_i$ : initial freezing point/ice crystallisation temperature. Adapted from Rahman et al. (2003).



## 5 Quality-related changes in frozen fish muscle

The quality of fish products after freezing and frozen storage is affected by factors such as fish species, temperature and handling before slaughter, slaughtering stress, the biological status of the fish, temperature of the pre-rigor storage, freezing rate, frozen storage temperature and time, temperature fluctuations, thawing procedure and prevention against oxidation (light and oxygen) (Sørensen *et al.*, 1995; Sigholt *et al.*, 1997; Erikson *et al.*, 1997; Kristoffersen *et al.*, 2006; Nielsen and Jessen, 2007). If fish is frozen quickly, stored at low, non-fluctuating temperatures and thawed in the best way according to its rigor-state, the quality can be as good as or better than fresh fish stored for a few days at 0 °C (Cappeln *et al.*, 1999). For optimally handled cod, the quality remains as high as for fresh cod for one month at -30 °C. The fish is still suitable for consumption after one year, though the characteristic frozen storage flavour starts to develop after approximately three months. Fatty species, such as trout, are stable during frozen storage and are still suitable for consumption after 18 months, though only of high quality up to six months. Due to a high content of polyunsaturated fatty acids (PUFA), fatty species are susceptible to lipid oxidation, which results in rancid taste and odour, if not packed in an oxygen-free atmosphere (Nielsen and Jessen, 2007). In lean fish, protein denaturation causes textural and functional changes in the fish muscle, whereas oxidative lipid degradation results in the characteristic cold-store flavour.

### 5.1 Factors affecting protein changes

Several factors are of importance in relation to the protein changes occurring during frozen storage: ice crystal formation, dehydration, increased concentration of salts in the unfrozen water pools, changes in lipids and fatty acids, lipid oxidation, enzymatic breakdown of trimethylamine oxide (TMAO) and interactions between these factors. Protein denaturation in frozen muscle has been reviewed by several authors (Love, 1970; Sikorski *et al.*, 1976; Shenouda, 1980; Mackie, 1993) and the main reasons are described in the following sections. An overview is given in Figure 3.

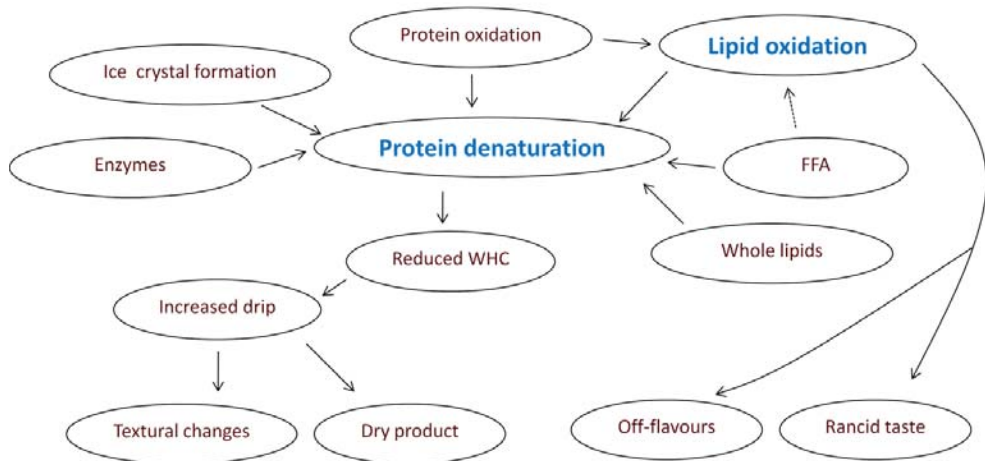


Figure 3. Quality-related changes in fish muscle during frozen storage.

### 5.1.1 Dehydration and the effect of inorganic salts

The removal of water from solution as a result of ice crystal formation leads to dehydration of the cells (see section 4.4) and the intra-cellular protein molecules. The three-dimensional structure of proteins is stabilised by a network of hydrogen bonds and as many of these are water mediated, they will be disrupted when water is removed. This can result in an exposure of hydrophilic and hydrophobic regions which can interact with other exposed regions, either in the same or in adjacent proteins, resulting in aggregation (Sikorski, 1978; Shenouda, 1980).

Increased solute concentration also affects protein denaturation and aggregation. Upon the freezing of fish muscle at  $-30^{\circ}\text{C}$ , potassium and sodium chlorides may form a solution of up to 7 % compared to about 0.5 % in the unfrozen muscle. These and other salt ions can interfere with secondary forces (electrostatic, van der Waals, hydrogen and hydrophobic) that stabilise the tertiary and quaternary structure of the proteins. At low ionic strength, many salts have a solubilising effect on proteins, but at higher ionic strength the inorganic salts compete for water with the hydrophilic groups. This may result in a salting-out effect and decreased solubility of the protein (Sikorski *et al.*, 1976; Shenouda, 1980). In a study by Buttkus (1970) aggregation of trout myosin in a frozen model system was higher at  $-10^{\circ}\text{C}$  compared to 0,  $-20$  and  $-30^{\circ}\text{C}$ . At  $-10^{\circ}\text{C}$  the concentration of salts was high and the salt ions were still dissolved in the water phase. Below  $-11^{\circ}\text{C}$ , which was the eutectic point of the model system (myosin-



potassium chloride-water solution), the rate of myosin aggregation decreased and reached a level similar to that at 0 °C at approximately -20 °C. The rates of lipid and protein oxidation as well as the activity of some proteases and lipases also increase to some point below the freezing point (Buttkus, 1970; Sikorski and Kolakowska, 1994). The low molecular weight peptides and free amino acids formed as a result of proteolytic activity may result in an undesirable flavour (Brown, 1986).

### **5.1.2 Changes in lipids and fatty acids**

Changes in lipids during frozen storage of fish can, directly or indirectly, lead to quality deterioration.

Fish and other seafood have a high content of PUFA, which are very susceptible to oxidation during frozen storage, and lipid oxidation is the main reason for quality deterioration in frozen stored fatty fish. Furthermore whole lipids, free fatty acids (FFA) and oxidised lipids or their products can interact with proteins, in some cases resulting in quality deterioration of especially lean species (Shenouda, 1980; Hultin, 1992; Mackie, 1993).

Due to lipid hydrolysis, FFA accumulate in the tissue during frozen storage, especially at high temperatures around -10 to -20 °C (Aubourg, 1999; Aubourg *et al.*, 2004; Rodriguez *et al.*, 2007). Slow freezing rates or fluctuating storage temperatures may result in the lysis of lysosomes and thereby increased activity of some endogenous lipases resulting in increased rates of FFA accumulation (Geromel and Montgomery, 1980). Accumulation of FFA does not in itself affect quality attributes of the product but have been shown to interrelate with lipid oxidation and have been proposed to have a pro-oxidant effect on lipids (Miyashita and Takagi, 1986; Han and Liston, 1987; Yoshida *et al.*, 1992; Aubourg and Medina, 1997; Rodriguez *et al.*, 2007).

Furthermore accumulation of FFA may lead to reactions between FFA and proteins resulting in decreased protein extractability. The exact mechanism of this interaction has not been shown, but is likely to be through electrostatic, Van der Waals, hydrogen or hydrophobic forces rather than covalent binding (Mackie, 1993).

The role of whole lipids on the stability of proteins is unclear as they have been suggested to have a protective as well as a detrimental effect (Mackie, 1993).

Oxidation of unsaturated fatty acids or triglycerides in fish results in the formation of free radicals produced through decomposition of lipid hydroperoxides via a free-radical mechanism. Free radicals can react with other molecules to form secondary products such as aldehydes, ketones, alcohols, short-chain fatty acids and hydrocarbons. Volatile carbonyl compounds are thought to be responsible for off-flavours and odours in oxidised seafood (Khayat and Schwall, 1983; Sikorski, 1994). Phospholipids undergo faster hydrolysis and oxidation than neutral lipids and though lean species only contain up to 2 % lipids, most of these are phospholipids, making them prone to oxidation despite the low lipid content (Han and Liston, 1987).

Free radicals can also contribute to protein denaturation and aggregation. Radicals may extract hydrogen from protein side chains such as SH groups resulting in protein radicals, which can react with other proteins or lipids to form aggregates. Malonaldehyde, propanal, and hexanal, which are the end products of lipid oxidation, may also react covalently with side chain groups of proteins (Mackie, 1993). Whether lipid and protein oxidation are concomitant processes or if one precedes the other is still unclear, though (Baron *et al.*, 2007).

### **5.1.3 Enzymatic breakdown of TMAO**

In gadoid species, such as cod and hake, TMAO can be enzymatically decomposed to formaldehyde and dimethylamine (DMA) by the action of trimethylamine-oxide aldolase (TMAOase, EC 4.1.2.32) leading to extensive textural changes (Babbitt *et al.*, 1972; Nielsen and Jørgensen, 2004). The rate of free formaldehyde and DMA production during frozen storage is typically highest around -10 °C and much lower at -30 °C. In a study by Howell *et al.* (1996) high-resolution NMR was used to detect DMA in cod samples stored at -20 °C for nine months, whereas samples stored at -30 °C for 12 months did not contain DMA in either the water- or salt-soluble extract. Formaldehyde is considered to have a higher spoilage potential than DMA, but the mechanism leading to tough, hard, fibrous and dry fish meat is not yet completely understood (Licciardello *et al.*, 1982; Leblanc and Leblanc, 1988; Chapman *et al.*, 1993). The reaction of formaldehyde with various chemical groups lead to denaturation/aggregation and possibly also cross-linking of proteins followed by decreased solubility, but the cross-linking theory has not been validated (Castell *et al.*, 1970; Babbitt *et al.*, 1972; Castell *et al.*, 1973; Tokunaga, 1974; Dingle *et al.*, 1977; Gill *et al.*, 1979; Leblanc and Leblanc, 1988; Chapman *et al.*, 1993; Mackie, 1993; Careche *et al.*, 1998; Careche *et al.*,

1999; Leelapongwattana *et al.*, 2008). Based on similarities in levels of protein denaturation and texture changes in cod (produces substantial formaldehyde) and haddock (produces negligible formaldehyde), some authors (Badii and Howell, 2002a; 2002b) suggest that formaldehyde is not a major factor responsible for muscle toughening in frozen stored cod and haddock fillets compared to lipid oxidation and ice crystal formation.

## **5.2 Changes in functional properties caused by protein changes**

Freezing and frozen storage may, as described above, result in denaturation and aggregation of especially myofibrillar proteins resulting in products with reduced WHC and increased drip loss upon thawing causing a hard, dry and fibrous fish product with altered colour and reduced juiciness (Sikorski *et al.*, 1976; Shenouda, 1980; Barroso *et al.*, 1998). The main changes are reported to occur in myosin light-chain, but actin and actinin also degrade during frozen storage (Careche *et al.*, 1998; Saeed *et al.*, 1999; Saeed and Howell, 1999; Kiran Jasra *et al.*, 2001; Badii and Howell, 2002b; Schubring, 2005; Kjaersgard *et al.*, 2006b). Some of the changes reported are increases in  $\beta$ -sheet at the expense of  $\alpha$ -helix structure (Herrero *et al.*, 2004). As the main part of muscle water is located within the myofibrillar structure, changes in this typically result in reduced WHC.

Numerous studies have shown a relationship between decrease in protein extractability and increased toughness of fish. Protein solubility and extractability are often used to characterize the degree of protein denaturation during frozen storage. Increased protein aggregation results first in an increased protein insolubility in salt solutions and thereafter in un-extractability in sodium dodecyl sulphate (SDS) and SDS plus  $\beta$ -mercaptoethanol. Storage temperature and time have great impact on the degree of protein denaturation and many authors have shown a relation between storage temperature, time as well as a combined effect and degree of protein denaturation or muscle toughness. Protein changes or changes in texture are reported to be higher after storage at -10 to -20 °C compared to -30 °C, temperatures below -30 °C are less studied (Licciardello *et al.*, 1982; Chapman *et al.*, 1993; Herrero *et al.*, 2004). Hsu *et al.* (1993) did, however, report that Pacific whiting fillets stored at -34 and -50 °C did not seem to be less denatured compared to fillets stored at -20 °C measured by salt-soluble protein extractability and  $\text{Ca}^{2+}$ -ATPase activity. Loss in SR  $\text{Ca}^{2+}$ -ATPase activity during frozen storage indicates changes in the sarcoplasmic reticulum membrane surrounding the

myofibrillar proteins. This has been used as an indicator of frozen storage denaturation (Godiksen *et al.*, 2003).

The effect of temperatures below -30 °C is primarily studied in relation to tuna quality, especially colour (Watabe and Hashimoto, 1986; Chow *et al.*, 1988) or with the use of a single temperature (typically -40 or -80 °C) used as a reference at which quality deteriorating processes are presumed to be halted or occur very slowly.

## 6 Experimental design, materials and methods

This chapter presents the different experiments carried out during the project. These are listed below (section 6.2) to give an overview and in the following sections sampling, handling and storage are described in detail. The different analyses used to determine quality-related changes during the storage of the fish and the reason for using these are described in detail in section 6.3.

### 6.1 Methodological considerations

The main focus of this study is the effect of various temperatures (-10, -20, -30, -40, -50, -60, -70 and -80 °C) on quality-related changes in the fish muscle during frozen storage. Therefore it was important to store the fish at as many temperatures as possible and using a variety of analytical methods to cover different aspects of quality deterioration. It was also of high importance to use a paired experiment with regard to temperature and thereby be able to analyse the same individual after storage at the different temperatures. The drawback of this design was, however, that it was only possible to use two replicates (two fish) at each temperature, and it was necessary to use different individuals from month to month in the ‘main experiment’, described below. As a consequence of this it was decided, by the end of the experiment, to set up two more storage experiments with cod and rainbow trout, using five replicates instead of two.

### 6.2 Experimental design

Below the nine different experiments carried out during the project are listed.

#### **Storage experiments:**

*Pilot experiment:* Effect of different freezing and thawing rates on the quality of cod and rainbow trout fillets stored at -20, -30 and -64 °C for one or 2.5 months.

*Main experiment (Paper I and II):* Frozen storage of cod and rainbow trout fillets at temperatures between -10 and -80 °C (-10, -20, -30, -40, -50, -60, -70 and -80 °C) for one to 18 months.

*Tuna experiment (Paper II):* Storage of frozen tuna fillets at temperatures between -10 and -80 °C (-10, -20, -30, -40, -50, -60 and -80 °C) for two or three months.

*Hake experiment:* Storage of frozen hake fillets at -10 and -80 °C (-10, -20, -30, -40, -50, -60 and -80 °C) for one or three months; design and results are not shown.

*High quality (HQ) cod experiment:* Storage of pre- and post-rigor cod fillets at -30, -40, -50 and -80 °C for one or three months.

*Rainbow trout experiment:* Storage of rainbow trout fillets at -30, -40, -50 and -80 °C for two or six months.

### **DSC-experiments:**

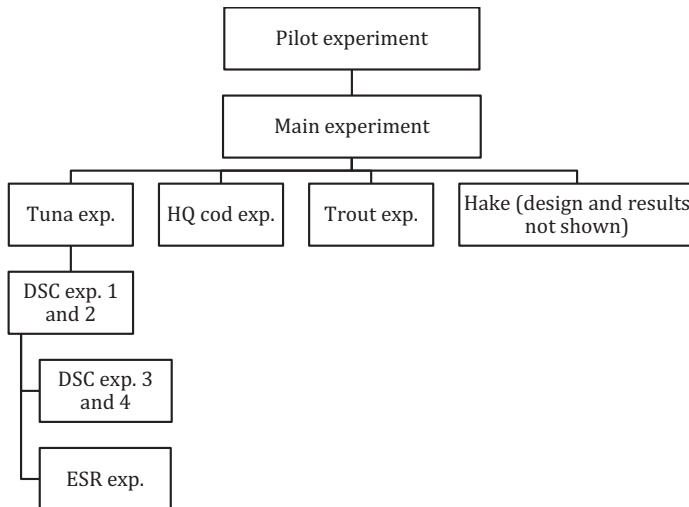
*Experiment 1 and 2 (Paper III):* Unfrozen water content in fresh tuna and pre-frozen cod.

*Experiment 3 and 4 (Paper III):* Ice crystallisation temperature in super-cooled fish muscle.

### **ESR-experiment:**

*ESR experiment:* Mobility of solutes in frozen cod muscle at various temperatures from -5 to -41 °C.

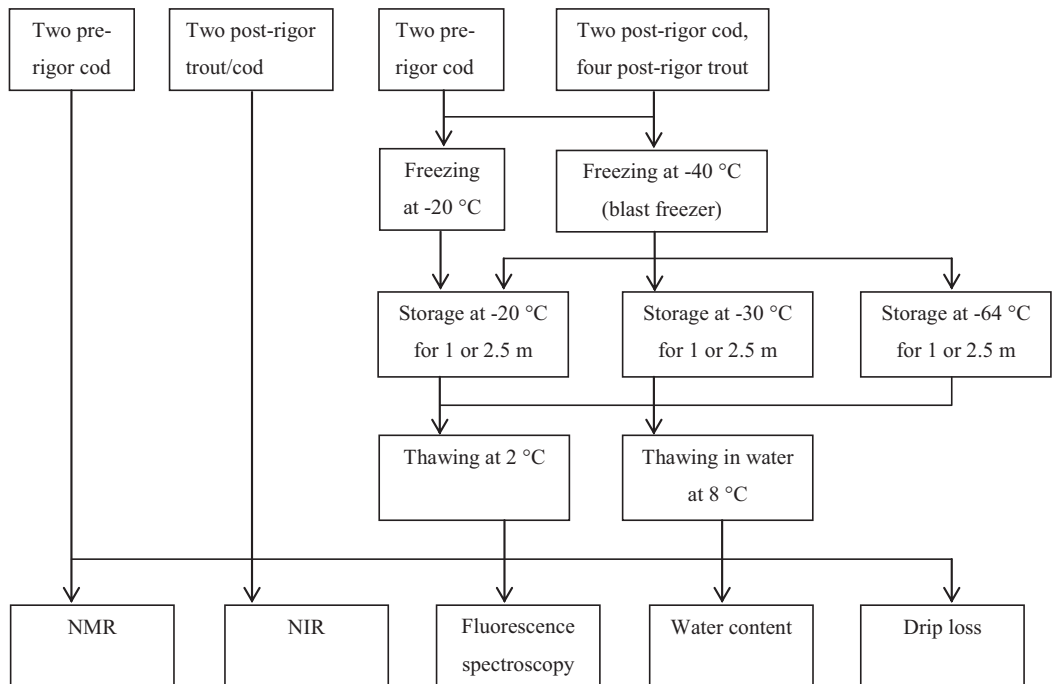
Figure 4 shows the chronological sequence of the experiments. The results of the pilot experiment lead to the design of the main experiment, which lead to new experiments with other species and more replicates. The tuna experiment was followed up by DSC experiments, the results of which lead to new DSC experiments and an ESR experiment.



**Figure 4.** Flow chart illustrating the chronological sequence of the experiments in the study.

### 6.2.1 Pilot experiment

Rainbow trout (*Oncorhynchus mykiss*) were obtained (May 2006) from a farm in southern Denmark, slaughtered and transported on ice to the National Institute for Aquatic Resources (DTU Aqua), where they were stored on ice for two days until rigor mortis had passed. Cod (Oresund) (*Gadus morhua*) were caught by a local fisherman (May 2006) and transported in a container with sea water to DTU Aqua immediately after catch. The flow chart in Figure 5 gives an overview of the design.



**Figure 5.** Flow chart showing the design of the pilot experiment. All samples were thawed over night in a 2 °C cold store.

Four cod were slaughtered, two were put on ice and analyses performed within a few hours and two were prepared for freezing as described below and frozen before the onset of rigor mortis. Six trout (3-4 kg each) and four cod (3 kg each) were filleted by hand on day three after slaughtering. Two trout and two cod were analysed on the same day. The middle parts of the fillets were cut in eight pieces each, vacuum packed, frozen in a -20 °C cold store or a -40 °C blast freezer. Fish frozen at -20 °C were stored at -20 °C and fish frozen at -40 °C were

stored at -20, -30 or -64 °C. Eight pieces from each fillet were distributed between these four different treatments and either thawed over night in a 2 °C cold store or in 8 °C running water. Pieces from the right and left fillet were stored for one and two and a half months, respectively. The following analyses were performed: low-field NMR spectroscopy, Near Infra Red (NIR) spectroscopy, fluorescence spectroscopy, water content and drip loss. The results of the study were used to decide which freezing and thawing methods to use in the main experiment.

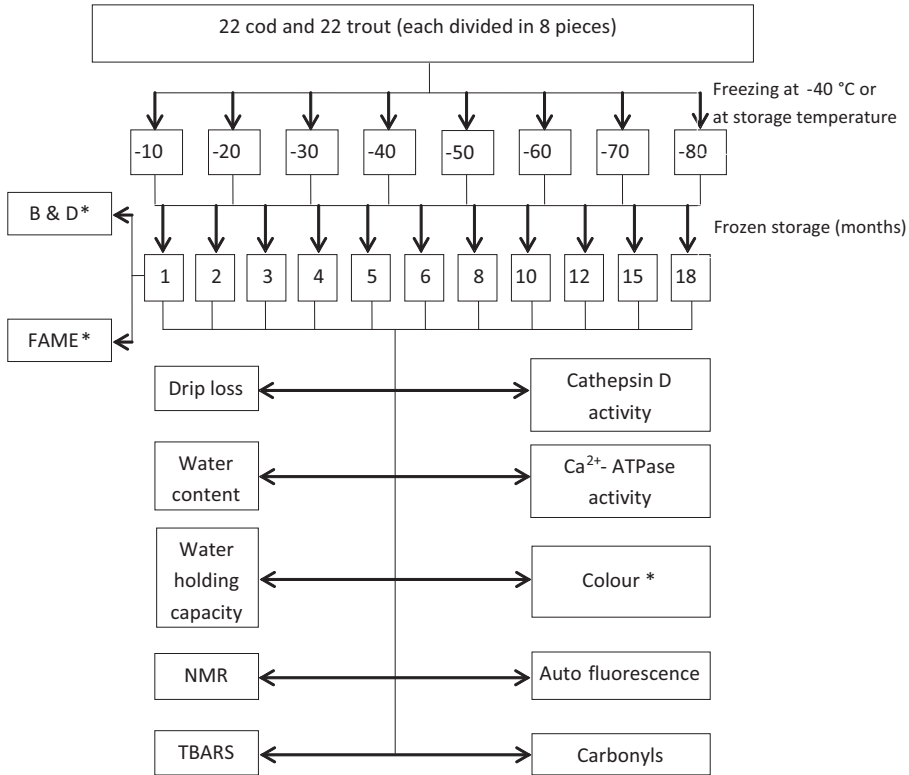
### **6.2.2 Main experiment**

An overview of the storage experiment is given in Figure 6. Rainbow trout (*Oncorhynchus mykiss*) were obtained (November 2006) from a farm in southern Denmark, slaughtered and transported on ice to DTU Aqua, where they were stored on ice for two days until rigor mortis had passed. North Sea cod (*Gadus morhua*) were caught in trawl in February 2007 and transported on ice to DTU Aqua maximum two days after catch. 22 trout (4-5 kg each) and 22 cod (5 kg each) were filleted by hand on day three after slaughtering. The loin parts of the fillets were cut in 20 pieces each and vacuum packed. The trout samples were randomly distributed to eight different freezers at temperatures between -10 and -80 °C (10 °C intervals) and frozen at the storage temperature. The cod samples were frozen in a -40 °C blast freezer and randomly distributed to the eight different freezers. The fish were stored from one to 18 months (1, 2, 3, 4, 5, 6, 8, 10, 12, 15 and 18). In order to investigate the specific effect of different storage temperatures statistically, a paired experimental design was used. The 40 pieces from each fish were stored at eight different temperatures but only for one storage period, and for each storage period, two different fish were examined. Thus, all samples stored at the eight different temperatures for a certain time originated from the same two fish. The fillets were cut in 20 pieces each instead of four to make it possible to thaw samples for different analyses at different days.

After each storage period the specimens were thawed in a 2 °C cold store over night (samples for drip loss, water content, WHC, NMR, auto fluorescence and colour) or in 8 °C water (samples for the other analyses) and the following analyses were performed: NMR measurements, content of thiobarbituric acid reactive substances (TBARS), drip loss, WHC, water content, colour measurements and activity of cathepsin D and SR Ca<sup>2+</sup>-ATPase. Lipid content (Bligh and Dyer) and fatty acid composition (FAME) were measured on samples



stored for one month at -80 °C only. For some of the analyses, fish fillet was minced for 5 seconds in a meat mincer (Foss Tecator Knifetec 1095 Sample Mill, Hillerød, Denmark).



**Figure 6.** Flow chart showing the design of the main experiment. Each of the eight pieces from the fish were further divided in five samples for different analyses. Samples for enzyme activity measurements, carbonyls and TBARS were thawed in water (8 °C) and other samples in a 2 °C cold store.

**\*)** B & D and FAME was measured after 1 month of frozen storage at -80 °C, colour was measured after 5, 8, 10, 12, 15 and 18 months in cod and after 8, 10, 12, 15 and 18 months in rainbow trout.

### 6.2.3 Tuna experiment

One 13.4 kg yellowfin tuna (*Thunnus albacares*) were obtained from Vagn P. Fisk (Næstved, Denmark). 28 pieces (30-40 g) were cut from the muscle, vacuum packed and frozen at -40 °C in a blast freezer (see Figure 7). The samples were stored at -10, -20, -30, -40, -50, -60 and -80 °C for two or three months and thawed in a 2 °C cold store overnight before analyses. The following analyses were performed: NMR, TBARS, WHC, water content, drip loss and colour measurement. Lipid content was determined using Bligh & Dyer.

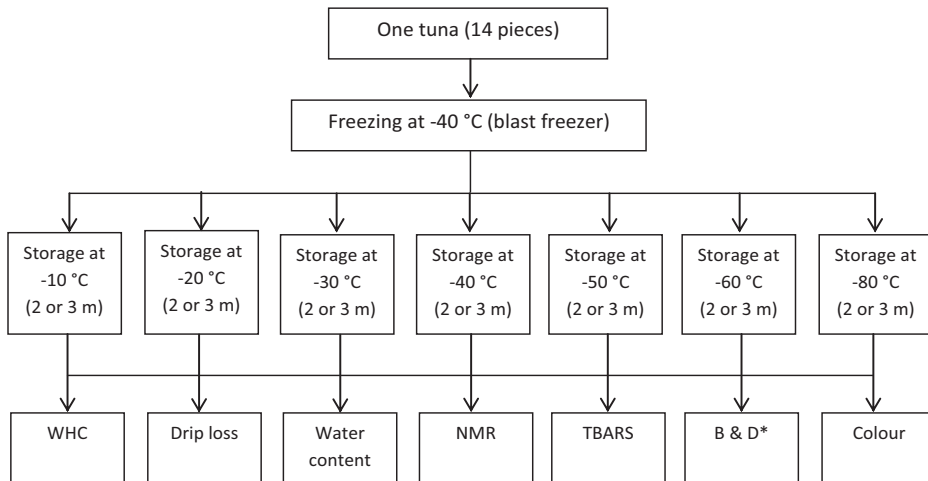


Figure 7. Flow chart showing the design of the tuna experiment. Samples for B & D and TBARS were thawed in water (8 °C) and samples for the other analyses in a 2 °C cold store.

\*) B & D was measured after 1 month of frozen storage at -80 °C.

#### 6.2.4 High quality cod experiment

10 North Sea cod (*Gadus morhua*) were obtained from a local fisherman a few hours after catch (See Figure 8).

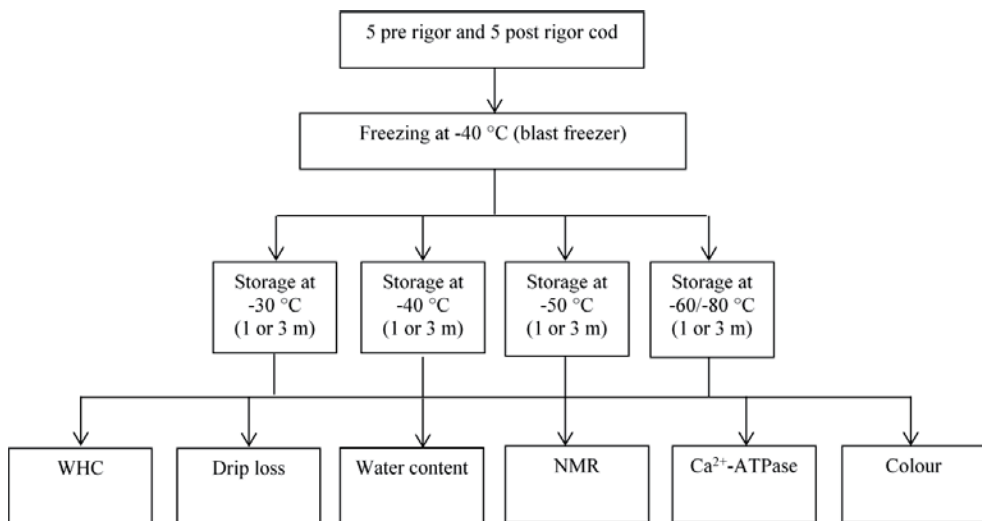


Figure 8. Flow chart showing the design of the HQ cod experiment. Samples for Ca<sup>2+</sup>-ATPase were thawed in water (8 °C) and other samples in a 2 °C cold store.

The cod were inspected by trained personnel and were all of high quality. The cod were slaughtered and the five specimens regarded as of highest quality (HQ cod) were each cut in eight pieces, vacuum packed and frozen in a -40 °C blast freezer, see Figure 8. The five other cod (reg. cod) were placed on ice for three days until rigor mortis had passed, packed and frozen as described above. The eight pieces from each fish were distributed at -30, -40, -50 and -60 °C and stored for one or three months. For practical reasons samples stored at -60 °C were moved to a -80 °C freezer after one month of frozen storage. Samples were thawed overnight at 2 °C in a cold store or in running water at 8 °C (only HQ samples stored for three months at -50 °C and reg. cod samples stored for three months at -40 and -50 °C). The following analyses were performed: NMR, WHC, dry matter content, drip loss, Ca<sup>2+</sup>-ATPase activity and colour measurement.

### 6.2.5 Rainbow trout experiment

Five rainbow trout (*Oncorhynchus mykiss*) were obtained from a farm in Southern Denmark and held in containers. After 12 months the trout were slaughtered, bled in water and cut in six pieces, vacuum packed and frozen in a -40 °C blast freezer. After freezing the six samples from each fish were distributed at -30, -40 and -50 °C for two or six months' storage as illustrated in Figure 9. The samples were thawed in running water and TBARS measured.

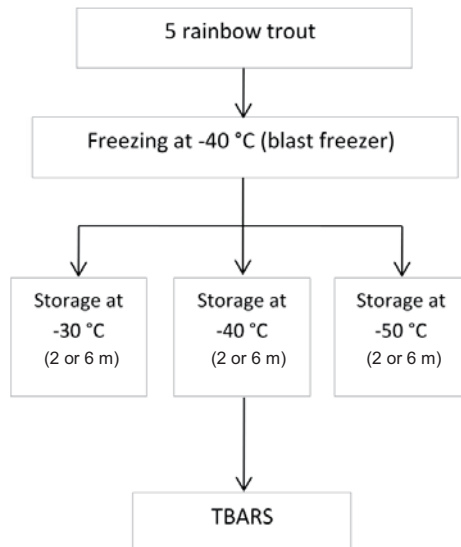


Figure 9. Flow chart showing the design of the rainbow trout experiment. Samples were thawed in water (8 °C).

### 6.2.6 DSC-experiments

Table 1 gives an overview of the four different DSC experiments referred to in the text below. One 11.3 kg yellow fin tuna (*Thunnus albacares*) were bought from Vagn P. Fisk (Denmark) filleted and four steaks cut from the muscle (used in DSC experiment 1). Samples were cut from one steak, prepared as described in section 6.4.8 and analysed the same day. The other steaks were vacuum packed and stored on ice for measurements on the three following days. The samples were measured in quadruple and in randomised order. Samples from the 11.4 kg tuna also used in the tuna experiment were vacuum packed, frozen in a blast freezer at -40 °C and stored at -40 °C for two months (used in DSC experiment 3). After thawing, half of the samples were prepared as described in section 6.4.8 and measured during the day. The rest of the tuna meat was stored on ice overnight and measured the following day.

**Table 1. DSC protocols used in the four experiments.**

DSC exp.	Samples	Scanning rate (°C/min)	Annealing time (min)	Annealing temperature
1	Fresh tuna	10	30	-10, -25, -35, -47, -60, -80
2	Pre-frozen cod	10	20, 30, 60, 120, 180, 240	-20, -60
3	Prefrozen tuna and herring, fresh and pre-frozen cod	1 or 10	10 or 30	-10, -12, -14, -16, -18, -20, -22
4	Fresh and pre-frozen cod	1 or 10	Up to eight hours	-5, -8, -10, -12, -14, -16, -20

In DSC experiment 2, farmed cod (*Gadus morhua*) from the Faroe Islands were frozen and stored at -40 °C until use. After thawing in running water, 12 samples from a single, randomly chosen fish were prepared as described in section 6.4.8. Five samples were measured on the day of preparation and the rest of the samples were stored at 2 °C for one day. The samples were measured in randomised order to avoid influence from the storage time.

Cod (*Gadus morhua*) from Oresund for DSC experiment 3 and 4 were obtained from a local fisherman and filleted on the day of catch. One fillet (DSC experiment 3) was stored on ice at 2 °C and analysed during the following three days. The other fillet was vacuum-packed (12 mbar), frozen in a blast freezer at -40 °C and stored at -40 °C for two months. The frozen

samples were thawed at 2 °C and subsequently stored on ice for up to three days while the samples were analysed.

Herring (*Clupea harengus*) for DSC experiment 3 were stored at -30 °C before thawing and preparation for analyses. After thawing they were stored on ice for up to three days. All measurements in DSC experiment 3 were made in duplicate. Frozen samples for DSC experiment 4 were thawed and analysed on the same day.

### 6.2.7 ESR experiment

All ESR measurements were conducted on fresh cod purchased from a local retailer and stored at 5 °C for up to three days.

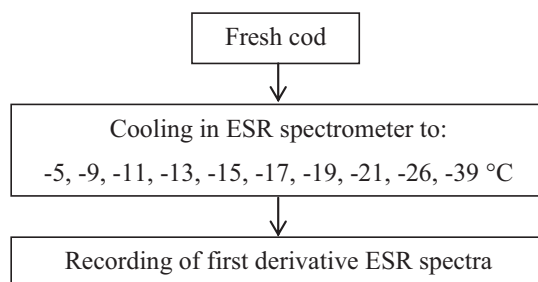


Figure 10. Flow chart showing the design of the ESR experiment.

### 6.3 Choice of methods

Analysis methods for the main experiment were chosen in an attempt to cover physical as well as chemical changes occurring in the fish during frozen storage. It was decided to test the use of the following analyses as indicators of quality changes during frozen storage: Drip loss, WHC, water content, fluorescence spectroscopy, NMR spectroscopy, NIR spectroscopy, TBARS, carbonyl group determination, measurement of colour (CIE L\*, a\*, b\*) and activity of the enzymes SR Ca<sup>2+</sup>-ATPase and cathepsin D. These analyses should provide information about the degree of textural deterioration, protein oxidation, protein denaturation, lipid oxidation, membrane integrity and water distribution in the fish muscle during storage.

The purpose of using the different methods is described in the following and thoroughly descriptions of the analyses are given in section 6.4.

*WHC*: WHC gives information about the property of the muscle to hold its own fluid. This property depends on the structure of the muscle proteins that bind and interact with the water molecules and is therefore influenced by protein changes.

*Drip loss*: Freezing of muscle foods typically lead to drip upon thawing because the protein network, cell membranes etc. are affected or to some degree disrupted by the formation of ice crystals, thereby altering the WHC.

*NMR*: The interaction between water and proteins is essential for the properties of the muscle. NMR relaxation measurements can give information about changes in the water distribution in the muscle during frozen storage. It can also be used to determine the number of water pools in the muscle based on differences in the water proton mobility. NMR and WHC typically correlate.

*DSC and ESR*: DSC measures changes in heat capacities caused by phase transitions such as freezing, melting, glass transition and protein denaturation. In this study DSC was used to determine the freezing point and the content of unfrozen water in fish muscle at different temperatures. ESR was used to measure the mobility in the unfrozen water phase of the muscle at different temperatures.

*TBARS*: Secondary lipid oxidation products (primarily aldehydes and ketones) produced by degradation of hydroperoxides (products of autoxidation of PUFA) give fish products with oxidised lipids the characteristic unpleasant flavour. Many of the carbonyl compounds react with thiobarbituric acid and the content of TBARS is therefore used as a measure of secondary lipid oxidation.

*Carbonyl group determination*: The presence of carbonyl groups in amino acid residues of proteins is taken as a presumptive evidence of oxidative modifications of the proteins (Levine *et al.*, 1990; Levine *et al.*, 1994). Therefore the reaction of modified proteins with carbonyl specific reagents provides a method for detection and quantification of protein oxidation.

*Fluorescence spectroscopy*: Aromatic amino acids are natural fluorophores and their emission is called auto fluorescence. Usually the aromatic amino acids are associated with the inner, hydrophobic part of the protein, but when the protein denature they become more exposed resulting in increased auto fluorescence. Fluorescence spectroscopy can therefore indirectly measure protein denaturation. Due to methodological problems, data from the fluorescence measurements are not valid and are therefore not presented or discussed.

*Enzyme activity measurements:* Enzyme activity measurements can be used to determine if the natural environment of the enzyme has changed, i.e. if for example a membrane has been destroyed during frozen storage. It was decided to measure the activity of an enzyme located within a membrane (SR  $\text{Ca}^{2+}$ -ATPase) and an enzyme located in the lysosomes (cathepsin D). In combination the activity of these enzymes should provide information about the integrity of membranes after freezing and frozen storage. The activity of a third group of enzymes (metallo proteinases) located between the muscle cell and the connective tissue, was also measured, but due to methodological problems the analysis was skipped a few months into the main experiment. This analysis could have given information about structural changes in the extra-cellular matrix.

*Colour measurements:* Colour is an important quality attribute for most foods. The colour of cod changes from a transparent white (in very fresh cod) to a milky white during frozen storage, whereas the trout typically loses colour because of breakdown of colour pigments. The colour of tuna is of high economic importance, because a shift from bright red to brown results in a downgrading of the quality of the product.

*Water content, B & D and FAME:* To provide information on the composition of the fish, oil content and fatty acid composition were measured after one month of frozen storage at  $-80\text{ }^{\circ}\text{C}$ ; water content was measured after each storage period.

It was decided not to use sensory evaluation of the frozen stored fish samples, because it is very expensive and time consuming. In addition a lot of sample material is required, which was not possible in this study.

## **6.4 Methods**

In the following the exact methods used to determine changes in different quality parameters during frozen storage are described. In general all analyses were made in duplicate unless otherwise indicated.

### **6.4.1 Drip loss, water content and water holding capacity**

Fillets were weighed before and after thawing, and the drip loss was measured as the loss in mass divided by the initial mass of the fillet. Before weighing, excess drip from the surface of the thawed fillets was wiped off using a paper towel.

The water content was measured as the loss in mass after drying 2 g sample overnight at 105 °C divided by the original sample mass.

The water holding capacity was defined as the capacity of the minced muscle to hold its own cellular fluid when centrifuged (Eide *et al.*, 1982). Portions of carefully mixed mince (2 g) were weighted into cylindrical tubes whose bottom was a filter allowing liquid but not solid material to pass. The tubes were then placed in 50 ml centrifuge tubes equipped with approximately 40 g glass marbles to provide space for the filtrate. Centrifugation was performed at 10 °C for 5 min at 1500 g in a Sigma 4K15 centrifuge equipped with a 12166 rotor. The WHC was expressed as the mass of water remaining in the muscle sample after centrifugation divided by the mass of water in the original sample.

#### **6.4.2 Low field NMR spin-spin relaxation measurements**

The NMR-measurements were performed on portions of 2-3 g mince that were weighed in small cylindrical glass tubes fitting in the 18 mm NMR sample tubes. The samples were equilibrated for 30 min in an 8 °C water bath before measurements. Low-field NMR relaxation measurements were performed on a Maran Benchtop Pulsed NMR analyser (Resonance Instruments, Witney, UK) operating at 23.2 MHz and equipped with an 18 mm variable temperature probe head. Transverse relaxation was measured at 8 °C as described by Jensen *et al.* (2002) using the Carr-Purcell-Meiboom-Gill CPMG pulse sequence. The inter-pulse spacing  $\tau$  (tau) was 200  $\mu$ s and the number of data points acquired was 1024, spaced by  $4\tau$  (even echoes sampled).

#### **6.4.3 Enzyme activity**

The preparation of homogenates for measurement of enzyme activity is time consuming and therefore it was decided to use a homogenate prepared with a common buffer for all enzyme activity measurements. According to the pilot experiment this did not affect the activities measured compared to activities measured using the buffers optimised for each of the assays.

Homogenates for determination of  $\text{Ca}^{2+}$ -ATPase and cathepsin D activity were prepared from 3.0 g muscle cut into small pieces in 24 ml cold 10 mM Tris-HCl buffer (pH 7.0) containing 0.25 M sucrose. A Potter-Elvehjem apparatus was used to homogenise the suspension by 10 up and down strokes with a Teflon pestle at 750 rpm. Afterwards the homogenate was



centrifuged at 1000 g (4 °C) for 15 min and the supernatant was kept on ice or frozen at -80 °C until use.

The activity of  $\text{Ca}^{2+}$ -ATPase was measured using the method described by Godiksen and Jessen (2001), based on the work of Simonides and Hardeveld (1990). The activity of  $\text{Ca}^{2+}$ -ATPase was defined as the difference between liberated inorganic phosphate ( $\text{P}_i$ ) from ATP in the presence (total activity) and absence (basal activity) of free  $\text{Ca}^{2+}$ .

Cathepsin D activity in the homogenates was assayed using a slightly modified method of Yasuda *et al.*, (1999) based on the fluorescence of the (7-methoxycoumarin-4-yl)acetyl (MOCAc) moiety which is split from the synthetic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe~Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH<sub>2</sub> catalysed by cathepsin D. The substrate was prepared from 1.0 mg MOCAc-Gly-Lys-Pro-Ile-Leu-Phe~Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH<sub>2</sub> dissolved in 200  $\mu\text{l}$  acetate buffer (50 mM sodium acetate, pH 4.0) and 369  $\mu\text{l}$  dimethylsulfoxid (DMSO) and thereafter diluted 4 times in the same acetate buffer. The final concentration of DMSO in the assay was 1.28 % and the substrate concentration 200  $\mu\text{M}$ . 80  $\mu\text{l}$  acetate buffer with 1 mM EDTA was added to the wells of a black assay plate (Corning Incorporated, NY) and heated for 15 min in a fluorescence spectrophotometer (Shimadzu UV-160A) at 35 °C. 10  $\mu\text{l}$  of sample solution (supernatant from the centrifuged homogenate) and 10  $\mu\text{l}$  of substrate were added and the fluorescence of the cut substrate measured at an emission wavelength of 393 nm with excitation at 328 nm for 10 min. The slope of the curve fluorescence *versus* time was used as a measure of the activity of the enzyme.

#### 6.4.4 TBARS

TBARS was determined according to the method of Vyncke (1970) with the modification that the reaction was done at 90 °C instead of in boiling water.

#### 6.4.5 Colour

Colour parameters (CIE 1976, L\*, a\*, b\* colour space) were measured by a Minolta Chroma Meter CR-200 (Konica Minolta Sensing Inc., Japan). The values reported are average of triplicate measurements at two or three different measuring points on the flesh side of the fillet depending on its size.

#### 6.4.6 Lipid content and fatty acid composition

The lipid content of the fish samples was determined by the method of Bligh and Dyer (1959) slightly modified by reducing solvent volumes. Fatty acid methyl esters were prepared using an AOCS official method (Ce 2-66, 1998) and analysed by gas chromatography, also using an official AOCS method (Ce 1 b-89, 1998). A HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an Omegawax 320 column (30m × 3.2mm, 0.25 µm; Supelco, Bellefonte, PA, USA) and a flame ionization detector (Hewlett-Packard, Palo Alto, CA, USA) was used for the analyses. The fatty acids were identified by comparison with chromatograms of standards.

#### 6.4.7 Carbonyls

Protein carbonyls were measured as described by Levine *et al.* (1990; 1994). A fish sample (0.5 g) was homogenized in 10 mL Tris buffer (pH 7.4, 50 mM, 1 mM EDTA) containing 0.01 % (w/w) BHT. 100 µL of the homogenate was precipitated with 50 µL of TCA (100%, w/v), centrifuged (12600g, 3 min, Heraeus Biofuge Pico, Thermo Fischer Scientific) and the pellet incubated with 1 mL of dinitrophenylhydrazine (DNPH) in 2 M HCl, in the dark for 1 h. For each sample a blank incubated in 2 M HCl and without DNPH was run in parallel. The samples were precipitated with 50 µL of TCA (100%, w/v), and the pellets washed three times with 1 mL of thanol/ethyl acetate (1:1, v/v). The pellet was redissolved in 6 M guanidine chloride in 20 mM KH<sub>2</sub>PO<sub>4</sub>. Spectroscopy determination of carbonyl groups was performed at 370 and 280 nm (Shimadzu UV-160A spectrophotometer). The carbonyl content was calculated using the absorbance measurements at 370 and 280 nm.

#### 6.4.8 DSC measurements

Small (10-25 mg) samples were cut from the fish muscle, carefully avoiding connective tissue. The samples were weighed directly into aluminium pans (Perkin Elmer DSC pans, 30 µl), sealed and stored at room temperature for at most 6 hours until measured. (Earlier experiments have shown that no measurable changes of the samples take place during this period.) Samples for experiment 2 were prepared as described above, but as it was not possible to measure 12 samples in one day, samples for the two following days were stored at 2 °C. When these samples were taken from the cold store before measurements, they were placed in a desiccator until they reached room temperature.

A differential scanning calorimeter (Perkin Elmer DSC 7) was used to determine the freezing point and the percentage of frozen water in the fish samples. The calorimeter was calibrated using a sodium chloride solution (10 % w/w and  $T_e = -21.12$  °C) for temperature calibration and indium ( $T_m = 156.60$  °C and  $\Delta H_s = 28.45$  J/g) for temperature and enthalpy calibration. An empty aluminium pan was used as reference and liquid nitrogen as coolant. The area of the endothermic melting curve,  $\Delta H$  (sample), and the relative water content,  $w_t$ , were used to determine the relative amount of unfrozen water,  $w_u$ , in the samples using the formula:

$$w_u = 1 - \frac{\Delta H(\text{sample})}{m_s \cdot w_t \cdot \Delta H_s(\text{ice})}$$

where  $m_s$  is the sample mass, and  $\Delta H_s(\text{ice})$  is the specific enthalpy change of ice melting, set to 334 J/g.

Experiment 1: The samples were equilibrated, held at 20 °C for 1 min and then cooled from 20 °C to the annealing temperature at -10, -25, -35, -47, -60 or -80 °C, annealed for 30 min and heated to 20 °C. The scanning rate was 10 °C/min in both directions.

Experiment 2: The samples were held at 20 °C for 1 min, cooled at 10 °C/min to the annealing temperature (-20 or -60 °C), annealed from 10 to 240 min and heated to 20 °C at 10 °C/min.

Experiment 3 and 4: The samples were scanned in the interval between -5 and -22 °C in order to see at what temperature a portion of the water in the muscle freezes. The following procedure was followed: The samples were equilibrated at 20 °C (experiment 3) or 1 °C (experiment 4) for 1 min, cooled to the annealing temperature at 1 or 10 °C/min, annealed from 10 min to several hours and finally heated to 20 °C at 10 °C/min. Unfrozen samples were recognised when their thermogram lacked a melting peak. In a special case, the samples were scanned from 1 to -2 °C, held for 2 min, cooled 2 degrees, held for 2 min and so on until reaching the annealing temperature at -12 or -14 °C.

Table 2 lists the different combinations of scanning rate and annealing time and temperature used in the four experiments.

**Table 2.** Scanning rate, annealing temperature and annealing time in the DSC-experiments.

Exp.	Samples	Scanning rate (°C/min)	Annealing time (min)	Annealing temperature (°C)
1	Fresh tuna	10	30	-10, -25, -35, -47, -60, -80
2	Prefrozen cod	10	20, 30, 60, 120, 180, 240	-20, -60
3	Prefrozen tuna and herring, fresh and pre-frozen cod	1 or 10	10 or 30	-10, -12, -14, -16, -18, -20, -22
4	Fresh and pre-frozen cod	1 or 10	Up to eight hours	-5, -8, -10, -12, -14, -16, -20

#### 6.4.9 ESR measurements

The spin probe TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) was dissolved to a concentration of 2 mM in a phthalate buffer (0.050 M, pH 5,8). Small cubes of cod muscle (5x5x5 mm) were placed in the buffered spin probe solution for approximately one hour at 5 °C.

Small samples (~5 mg) were cut from the cubes avoiding the outer layer (1 mm) and placed between two layers of plastic foil (NEN 40 HOB/LLDPE 75, Danisco Flexible, Lyngby, Denmark) that were sealed with a T300 table sealer (PM Pack Service, Horsens, Denmark). The first derivative ESR spectra were recorded using a Bruker ECS 106 X band ESR spectrometer in the temperature interval from -5 to -41 °C. The general settings of the Bruker were as follows: Microwave power: 0.2 mW, modulation frequency: 100 kHz, modulation amplitude: 1.019 Gauss. Time constant and conversion time varied. The method is described in detail by Hansen *et al.* (2003; 2004).

#### 6.4.10 Data analysis

Univariate one- and two-way analyses of variance (ANOVA) were used to look for possible differences in quality parameters due to temperature or time. According to the experimental designs in the storage experiments, repeated measures ANOVA was used between groups at different temperatures and a Bonferroni post test was used to compare all pairs of columns. Test for linear trend was made using regression on the temperature variable. The multivariate analysis methods principal component analysis (PCA) and partial least squares (PLS) regression were applied to the NMR relaxation curves with data from samples as rows in the

$X$ -matrix and (for the PLS regression) WHC or drip loss as  $Y$ . Each column in the data matrix was mean-centred. The multivariate models based on data from the main experiment were cross-validated using 2 segments, each with 88 members (samples from 11 different fish, stored for 11 different periods at eight temperatures in each segment). Multivariate models based on data from the other experiments were fully cross-validated.

GraphPad Prism<sup>®</sup> v4.03 (GraphPad Software Inc.) was used for univariate one- and two-way analysis (ANOVA) including Bonferroni post test and The Unscrambler<sup>®</sup> v9.1 (Camo Process A/S) was used for PCA and PLS regression.



## 7 Results and discussion

The results of the main experiment are covered thoroughly in Paper I and II and results of the DSC measurements are covered in Paper III, but in order to increase the readability of the report, the results are also presented in this chapter. The results of the pilot experiment were used in the design of all the other storage experiments and are therefore presented separately in the first section. In the following sections first the results of the different storage experiments including unpublished results and afterwards the results of the DSC and ESR experiments are presented and discussed in relation to each other. Most tables from the papers are referred to in the text, but not repeated. When referring to the results of 1-way ANOVA in relation to tables or figures, the values from the 1-way ANOVA are not shown; instead the results of 2-way ANOVA and 1-way ANOVA for each month are given. In section 7.2.8 quality-related changes during frozen storage of fish reported by other authors and the collected results of the storage experiments of this project are summarised in Table 5 and Table 6.

### 7.1 Pilot experiment

The pilot experiment was set up to analyse the effect of different freezing, storage and thawing methods on selected quality parameters in rainbow trout and pre- and post-rigor cod fillets. Only the results of drip loss and NMR relaxation measurements are reported.

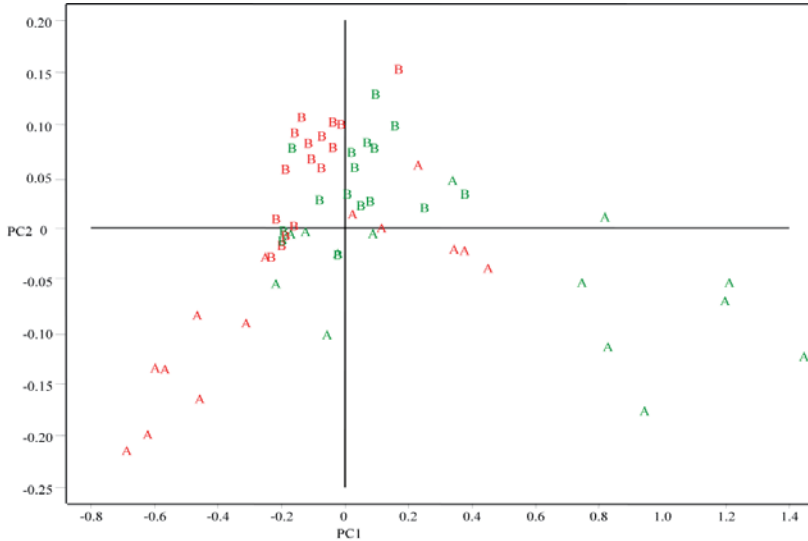
A PCA performed on the NMR relaxation data for cod showed that the samples were partly grouped according to rigor state before freezing and moreover the pre-rigor samples were also affected by thawing method as illustrated in Figure 11.

In general, pre-rigor fillets experienced a greater drip loss than post-rigor fillets and fast thawed (in 8 °C water) pre-rigor fillets experienced greater drip losses than pre-rigor fillets thawed slowly in a cold store (2 °C).

This is in agreement with Kristoffersen *et al.* (2006) who showed that cod filleted pre-rigor and stored at 4 °C for 10 days had a higher drip loss compared to post-rigor filleted cod. It is also well known that thaw rigor can result in a high drip loss.

In post-rigor fillets there was an effect of freezing as well as storage temperature on the drip loss. Post-rigor fillets frozen at -20 had a higher drip loss than fillets frozen at -40 °C

regardless of storage temperature, and fillets stored at -20 had a higher drip loss than fillets stored at -40 °C.



**Figure 11.** Scores plot of PC 1 (principal component 1) vs. PC 2 from a PCA with NMR relaxation measurements on thawed cod fillets. Explained X-variation were 96 % for PC 1 and 4 % for PC 2. Symbols and colours used: A, pre-rigor; B, post-rigor; red, thawed in water (fast thawing); green, thawed in a 2 °C cold store (slow thawing). Each point is positioned in the centre of its label.

A PCA on the NMR relaxation data showed that thawing method appeared to have an effect on rainbow trout. This effect did not have any relation to differences in the drip loss, but was probably related to the WHC that has been shown to correlate with NMR relaxation measurements (Trout, 1988; Jepsen *et al.*, 1999; Andersen and Jørgensen, 2004). Freezing rate was expected to affect the water distribution and drip loss in the trout samples, but neither freezing method nor storage temperature seemed to have an effect on trout samples during two months of frozen storage.

The results of the pilot experiment were used in the design of the main experiment with regard to freezing and thawing method.

## 7.2 Storage experiments

Many of the tables referred to in the text are located in the papers; statistical methods and *p*-values are also given in the papers. In this section the results of the four storage experiments are given. When needed to make the text clearer, sub-headings are used to separate the results



of the different experiments; otherwise the results of the different experiments are presented and discussed consecutively. Figure 12 gives an overview of the main results of the storage experiments.

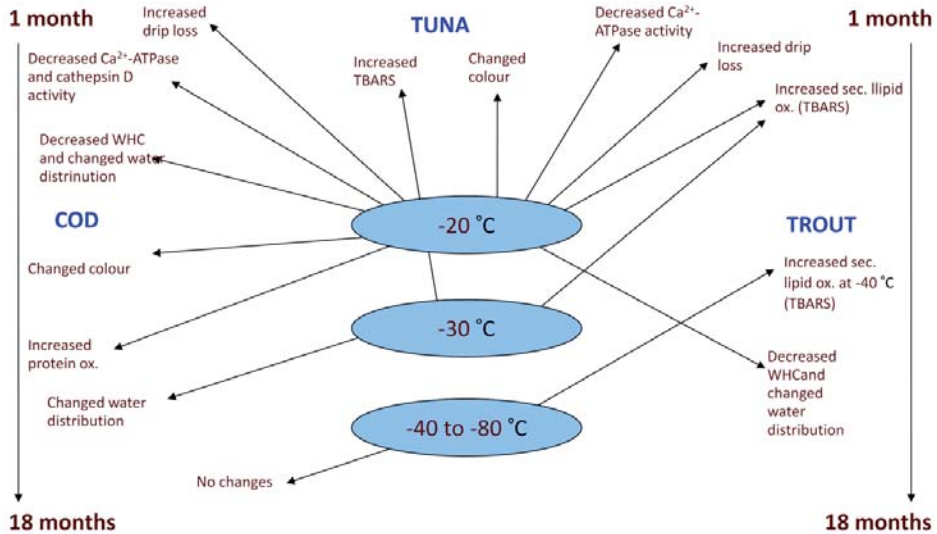


Figure 12. Various effects of temperature on quality of fish stored at -20 to -80 °C for 1-18 months.

### 7.2.1 Composition

The average lipid content of the 22 cod used in the main experiment was  $0.7 \pm 0.1$  % and the water content was  $79.7 \pm 1.3$  %. There were no significant differences in the PUFA composition of the 22 cod (1-way ANOVA,  $p \gg 0.05$ ). The average lipid content of the 22 trout (main experiment) was  $8.2 \pm 1.3$  % and the water content was  $72.7 \pm 0.1$  %. There were no significant differences in the PUFA composition of the 22 trout (1-way ANOVA,  $p \gg 0.05$ ). The average lipid content of the tuna used for the tuna experiment was  $0.9 \pm 0.2$  % and the water content was  $72.9 \pm 0.4$  %. The average water content of the five HQ cod was  $80.6 \pm 0.9$  % and of the reg. cod  $81.1 \pm 0.7$  %. The lipid content was not measured.

### 7.2.2 Effect of temperature on drip loss and water holding capacity

#### *Main experiment, cod*

In the main experiment, the drip loss of cod was highest for samples stored at high temperatures and decreased with decreasing temperature ( $p < 0.0001$  in the test for linear trend, table 1, Paper I), and generally samples stored at -10 and -20 °C had a higher drip loss than samples stored at lower temperatures. The WHC during storage at -10 to -80 °C for one to 18 months is shown in table 2, Paper I. The WHC of samples stored at -10 and -20 °C decreased during frozen storage and, as expected, samples stored at -10 °C had lower WHC compared to all other temperatures at all times except for one of the samples stored in a broken bag at -80 °C for four months. After five months and during the rest of the experiment (except at 8 months) samples stored at -20 °C had significantly lower WHC than samples stored at -30 to -80 °C. No difference was found between WHC in samples stored at temperatures from -30 to -80 °C during the time of the experiment. Similar results were reported by Schubring (2004; 2005), who found decreased WHC for cod samples stored up to 10 months at -10 and -20 °C but no changes in samples stored at -30 °C, the decrease in WHC for samples stored at -10 and 20 °C correlated with storage time. Mørkøre and Lilleholt (2007) also reported a higher drip loss in cod frozen and stored at -10 and -25 °C compared to -40 to -70 °C. Their cod were only stored for 10-11 days, so the temperature during freezing probably had a more pronounced effect than the storage temperature.

The increased drip loss and decreased WHC for samples stored at -10 and -20 °C is possibly a result of protein changes leading to altered functional properties. Reduced WHC is primarily due to denaturation/aggregation of actin and in particular myosin. These are the main contractile proteins responsible for functional properties and their denaturation and aggregation is typically caused by ice crystal growth, increased ionic strength due to water crystallisation and protein and lipid oxidation (Sikorski *et al.*, 1976; Shenouda, 1980; Mackie, 1993; Nott *et al.*, 1999). Another factor could be formation of DMA and formaldehyde from trimethylamine-N-oxide (Nielsen and Jørgensen, 2004) as formaldehyde may form cross-links between proteins resulting in decreased protein solubility, aggregation and thereby loss of functional properties (Castell *et al.*, 1970; Babbitt *et al.*, 1972; Sikorski, 1978; Gill *et al.*, 1979). The rate of DMA formation has been shown to decrease in the temperature interval

from -10 to -30 °C by numerous authors e.g. (Gill *et al.*, 1979; Leblanc and Leblanc, 1988; Chapman *et al.*, 1993; Sotelo *et al.*, 1994; Badii and Howell, 2002a).

#### *Main experiment, rainbow trout*

The drip loss of trout was highest for samples stored at high temperatures and decreased with decreasing temperature (Test for linear trend  $p < 0.0001$ , table 1, Paper II) and generally samples stored at -10 and -20 °C had higher drip losses than samples stored at lower temperatures. The decrease in the WHC of trout during frozen storage was very similar to that seen in cod. Samples stored at -10 °C had a lower WHC than samples at all other temperatures after all storage times except one and three months (table 2, Paper II). After 12 months also samples at -20 generally had reduced WHC compared to samples stored at lower temperatures, but no differences in WHC were seen between samples stored from -30 to -80 °C.

#### *Tuna experiment*

The drip loss and WHC of tuna were measured after two and three months (table 3, Paper II). The drip loss was higher for samples stored at -10 compared to -20 to -60 °C, but there were no differences between samples stored at other temperatures or between samples stored for two and three months. After both storage times there was an increase in WHC with decreasing temperature (linear trend: 2 months,  $p < 0.001$ ; 3 months,  $p = 0.002$ ), but no significant differences in means between different storage temperatures (1-way ANOVA). Surprisingly, samples stored for three months had a significantly higher WHC compared to two months, which was not related to differences in drip loss. All WHCs were, however, very high (over 89 %).

#### *HQ cod experiment*

Drip loss and WHC in the temperature interval from -30 to -80 °C were studied further in the HQ cod experiment; values are shown in Table 3.

Moreover, pre-rigor cod fillets of very high quality were included in the study to see if these were more susceptible to the effect of different storage temperatures.

Five cod of high quality (pre-rigor) and five cod of regular quality (post-rigor) were stored at -30, -40, -50 and -60/-80 °C for one and three months. Most of the samples were thawed at 2 °C overnight (slow thawing) as this procedure was determined as the best for especially pre-

rigor cod in the pilot experiment. Many of the samples did, however, turn out to have high drip losses and therefore it was decided to thaw some of the three months cod in water (fast thawing) to see if this would decrease the drip loss.

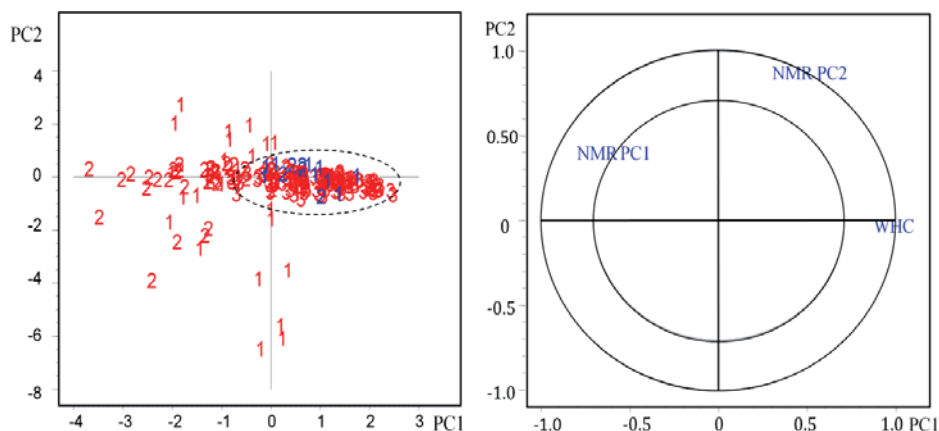
**Table 3.** Water holding capacity and drip loss of cod fillets frozen stored for 1 or 3 months.

Fish		Frozen storage (months)											
		WHC								Drip loss			
		Regular cod				HQ cod				Regular cod		HQ cod	
		1		3		1		3		1	3	1	3
	Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD					
1	-30	84.9	2.6	78.3	33.5	72.0	2.1	78.2	2.9	6.0	8.5	3.7	10.5
	-40	86.2	0.5	91.1	4.5	72.7	0.9	91.2	4.5	6.5	3.4	3.7	7.2
	-50	81.9	0.0	89.3	0.3	76.6	5.9	89.3	0.3	4.1	5.2	3.7	10.3
	-80	88.5	2.8	89.5	0.8	72.6	6.2	89.6	0.8	5.1	5.8	3.5	9.2
2	-30	74.4	7.5	72.8	2.0	64.5	2.0	73.1	2.0	5.1	7.9	21.3	5.0
	-40	74.4	2.2	87.6	4.9	77.7	0.4	87.7	4.9	6.2	4.0	18.9	
	-50	63.0	33.2	90.2	0.5	72.4	1.5	90.2	1.7	4.8	4.0	16.2	3.0
	-80	83.9	2.8	72.7	6.5	75.1	3.6	72.8	6.5		5.6	2.8	
3	-30	81.9	6.4	82.5	3.6	65.5	4.2	82.6	3.6	7.3	8.3	5.1	5.8
	-40	83.5	1.0	89.5	0.1	76.7	1.3	89.5	0.1	4.0	5.8	-1.3	3.9
	-50	85.7	0.5	89.2	0.8	77.2	4.2	89.2	0.8	6.8	5.6	4.0	7.2
	-80	86.6	3.6	85.0	0.7	67.0	0.6	85.2	0.7	5.3	5.0	2.1	3.5
4	-30	83.9	5.9	68.6	1.6	54.3	6.4	68.1	1.6	6.7	9.8	11.6	10.6
	-40	76.2	2.9	80.3	3.3	65.2	1.5	80.1	3.3	7.1	5.9	16.0	8.7
	-50	85.2	0.3	82.8	1.3	74.7	4.5	82.5	1.3	4.6	7.1	3.9	4.7
	-80	86.9	0.1	89.3	4.2	75.7	1.5	89.1	4.3	4.6	5.7	3.0	4.2
5	-30	80.0	4.0	81.9	0.6	51.3	6.1	81.4	0.6	8.4	9.1	7.1	8.4
	-40	82.6	0.5	70.5	19.0	58.5	7.3	69.5	19.7	4.8	5.1	6.0	6.5
	-50	90.1	4.2	87.5	0.9	71.4	8.6	86.9	0.9	5.2	5.0	4.3	12.6
	-80	85.2	5.1	86.0	1.7	67.8	0.3	85.7	2.1	7.0	7.4	5.0	5.1
1-way ANOVA		<i>p</i> = 0.25		<i>p</i> = 0.10		<i>p</i> = 0.01		<i>p</i> = 0.10		0.25	<i>p</i> < 0.0001	<i>p</i> = 0.11	<i>p</i> = 0.16

a) n=2.

The variation between samples thawed in water was less than the variation between samples thawed in air. Furthermore water thawed samples generally had higher WHC. Generally, there was no effect of temperature, rigor/quality state, individual or storage time (results not shown).

Figure 13 shows a scores plot with all samples stored at -30 to -80 °C from the main experiment and the HQ cod experiment as  $X$  and WHC, NMR PC 1 and 2 (extracted from a PCA on all NMR relaxation (-30 to -80 °C) data) as  $Y$ . In general the drip loss and WHC of the samples varied more than in the main experiment.



**Figure 13.** Left: Scores plot of PC 1 (principal component 1) vs. PC 2 from a PCA with PC 1 and 2 from a PCA on NMR relaxation measurements (on thawed cod fillets from the main experiment and the HQ cod experiment) and WHC (also from both experiments) as variables. Explained X-variation were 52 % for PC1 and 35 % for PC2.

Symbols and colours used: 1, reg. cod (from HQ cod exp.); 2, HQ cod (from HQ cod exp; 3, cod from the main exp. (all in the marked area); blue, thawed in water (fast thawing); red, thawed in a cold store (slow thawing). Each point is positioned in the centre of its label.

All samples from the main experiment (numbered 3) are crowded in the marked area characterised by high WHC together with samples from the HQ cod experiment thawed in water (blue). Nearly all the samples outside of the marked area are slowly thawed samples from the HQ cod experiment (numbered 1 and 2). Surprisingly the fast thawed HQ cod samples are very similar to the slowly thawed samples from the main experiment, whereas the slowly thawed samples from the HQ cod experiment differ from those. The positive effect on WHC of the fast thawing is contradictory to the results of the pilot experiment. The results do, however, show that different storage temperatures between -30 and -80 °C do not affect the WHC of cod of very high quality stored for up to three months.

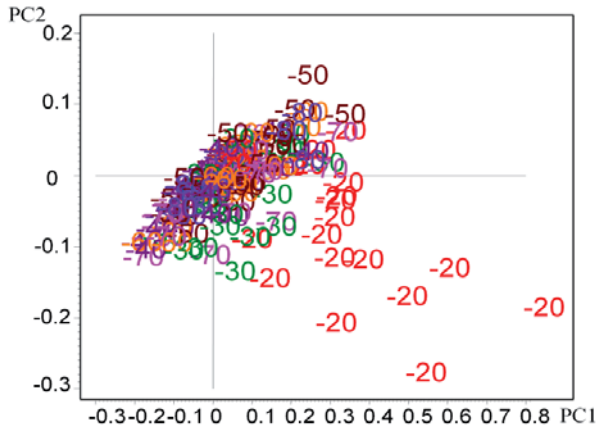
### 7.2.3 NMR

The NMR relaxation curves contain information on the water content and distribution in the muscle. By normalising the signal to common total amplitude, contributions from a trivial difference in water content is removed. Thus, changes in curve shapes reflect changes in water distribution only.

#### *Main experiment, cod*

A PCA showed that cod samples stored at -10 °C as expected were most different from other samples. When removing the -10 °C samples from the data set and redoing the PCA, also samples stored at -20 °C showed up as different from the rest (Figure 14).

However, they did not differ from samples stored at lower temperatures until after approximately 4-5 months of frozen storage. Steen and Lambelet (1997) showed that cod mince stored at -10 °C for 4 months had a larger proportion of slowly relaxing water compared to samples stored at -20 and -70 °C; moreover the proportion of slowly relaxing (loosely bound) water increased with increasing storage temperature. Jensen *et al.* (2003) also showed that for cod samples stored for at least three months, storage at -20 °C resulted in a higher proportion of slowly relaxing water compared to -30 °C. Large amounts of loosely bound, slowly relaxing water typically results in reduced WHC (Andersen and Jørgensen, 2004). In the present study, samples stored at -30 °C did not differ from samples stored at -40 to -80 °C for the first 10 months but after 12 months of frozen storage the -30 °C samples differed from those stored at lower temperatures. The change in water distribution as measured by the NMR technique for samples stored at -30 °C after 12 months is probably caused by textural changes affecting water distribution or binding.



**Figure 14.** Scores plot of PC 1 (principal component 1) vs. PC 2 from a PCA on NMR relaxation measurements on thawed cod fillets from the main experiment. Explained X-variation were 84 % for PC 1 and 15 % for PC 2. Each point is positioned in the centre of its label.

Steen and Lambelet (1997) showed very high correlations between NMR  $T_2$ -values and several analytical methods (instrumental and sensory) for determining texture of cod mince. Lean fish species are claimed to have a storage life (consumer acceptability) of at least 12 months at  $-30\text{ }^{\circ}\text{C}$  (Almandos *et al.*, 1984; Chapman *et al.*, 1993; Nielsen and Jessen, 2007), which is in fair agreement with the results of the NMR measurements in the present study. It was not possible to see any differences caused by storage temperature in samples stored at  $-40$  to  $-80\text{ }^{\circ}\text{C}$  during 18 months of frozen storage.

Using the NMR-relaxation curves as  $X$ -variables in a PLS regression, the WHC ( $Y$ -variable) of the samples were predicted (results not shown). Predicted and measured WHC correlated well ( $r = 0.86$ ,  $n=176$ ) considering an error of approximately 2.9 % in the WHC analysis and the results of the two methods were very similar. This relationship was also shown by Andersen and Jørgensen (2004) and Jepsen *et al.* (1999).

As described in section 0 differences in WHC and water distribution between HQ cod samples stored at different temperatures were mostly due to thawing method and not affected by storage temperature.

*Main experiment, rainbow trout*

The NMR relaxation data for rainbow trout were used as  $X$ -variables in a PCA. Samples stored at -10 °C were different from all other samples and when removing these from the data set also samples stored at -20 °C were different (Paper I, figure 1). Samples stored at -20 °C for 12-18 months were grouped away from samples stored at -30 to -80 °C, whereas most of the samples stored at -20 °C for 1-10 months were in the area between the two groups (plot not shown). This is in agreement with the results of the WHC of trout. It was not possible to see any differences between samples stored at temperatures from -30 to -80 °C. Samples stored at -20 °C did not begin to differ from samples stored at -30 to -80 °C until after 12 months of frozen storage and samples stored at -30 °C was not different (according to the NMR measurements) from samples stored at lower temperatures at any time. This was expected as trout is more robust to frozen storage compared to cod and as previously mentioned trout and other fatty species have a storage life (consumer acceptability) of at least 18 months at -30 °C (Nielsen and Jessen, 2007).

The WHC of the samples was predicted using the NMR-relaxation curves as  $X$ -matrix and the measured WHC as  $Y$ -vector in a PLS regression. Predicted and measured WHC correlated well ( $r = 0.84$ ,  $n=2$ ).

*Tuna experiment*

There were no systematic differences between relaxation curves from NMR measurements on tuna samples stored at different temperatures for two to three months.

**7.2.4 Membrane stability measured as  $\text{Ca}^{2+}$ -ATPase and cathepsin D activity**

Cod and rainbow trout samples stored at -10 and -20 °C generally had significantly lower  $\text{Ca}^{2+}$ -ATPase activity than cod and trout samples stored at or below -40 and -30 °C respectively (1-way ANOVA, see Figure 15). Hsu *et al.* (1993) measured the  $\text{Ca}^{2+}$ -ATPase activity of actomyosin in Pacific whiting and reported a more rapid decrease of enzyme activity for samples stored at -8 °C compared to samples stored at -20, -34 and -50 °C. Godiksen *et al.* (2003) were able to differentiate fish stored at -30 (highest activity) and -20 °C after five months of frozen storage. This is in fair agreement with our results as some differences can be expected due to freezing and thawing method and variation between individuals. However, del Mazo *et al.* (1999) did not observe any significant differences



between actomyosin  $\text{Ca}^{2+}$ -ATPase activity in hake fillets stored at -20 and -30 °C for 49 weeks.

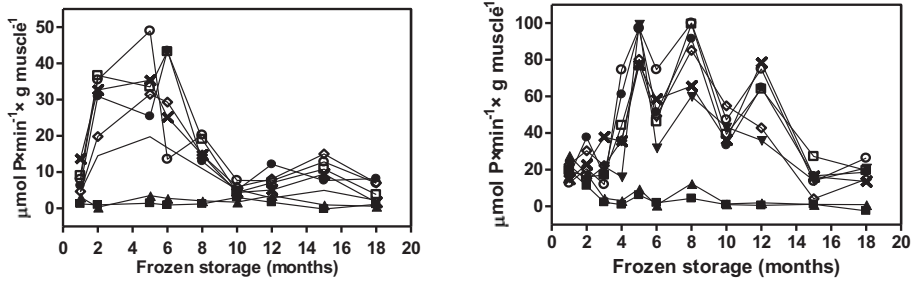


Figure 15.  $\text{Ca}^{2+}$ -ATPase activity in cod (left) and rainbow trout (right). Symbols used: ■, -10; ▲, -20; ▼, -30; ●, -40; ✕, -50; ◇, -60; ○, -70; □, -80 °C. 2-way ANOVA, cod: temperature,  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ ; all p-values are for analyses without -10 °C. 2-way ANOVA, trout: temperature,  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ ; -10 °C is excluded from the 2-way ANOVA.

The activity decreased significantly at both temperatures and no activity was detected from 36 weeks onwards, which was ascribed to denaturation of actomyosin. For cod as well as trout samples stored at temperatures between -30 and -80 °C in the main experiment, the enzyme activity increased dramatically after respectively two and four months of frozen storage. The reason why  $\text{Ca}^{2+}$ -ATPase activity in samples stored at -10 and -20 °C was not activated may be that the enzyme had become denatured or the SR membrane was disrupted. Inoue *et al.* (1992) measured  $\text{Ca}^{2+}$ -ATPase activity of frozen carp myosin and reported an increased activity for samples stored at -15 and -20 °C during the first 12 hours of frozen storage. The activity of samples stored at -5 to -13 °C decreased. Godiksen *et al.* (2003) also reported on activation of  $\text{Ca}^{2+}$ -ATPase activity as a result of freezing and suggested that the freeze-induced activation of the enzyme was due to changed interaction between  $\text{Ca}^{2+}$ -ATPase and the surrounding membrane lipids.

$\text{Ca}^{2+}$ -ATPase activity was also measured in the HQ cod experiment, but no effect of rigor-state or temperature was observed (results not shown).

During storage from 1 to 18 months, cod samples stored at -10 and -20 °C generally had significantly lower cathepsin D activities than samples stored at lower temperatures (table 3, Paper I). Storage temperature did not seem to affect cathepsin D activity in trout (table 4,

Paper II). According to Nilsson and Ekstrand (1995) frozen storage temperature affect lysosomal membrane integrity resulting in increased lysosomal enzyme leakage and thereby increased activity of  $\beta$ -N-acetylglucosaminidase for trout samples stored at -18 °C compared to -40 °C, this is contradictory to our cod results. Large variations in activity over time were observed in both cod and trout which might be due to variation between individual fish. Another possibility is that an increased activity caused by release of cathepsin D to the tissue from lysosomes as a result of destroyed membranes competed with a decreased activity over time due to protein denaturation and/or other protease activities. These two 'opposite' reactions might also explain the unclear results, which are very difficult to interpret in relation to the effect of temperature on protein denaturation/oxidation and membrane integrity.

### **7.2.5 Secondary lipid oxidation measured as TBARS**

Cod samples stored at -10 °C generally had higher TBARS content than samples stored at lower temperatures (1 way ANOVA, table 4, Paper I), but there were no differences between samples stored at -20 °C and lower. This is in fair agreement with Aubourg and Medina (1999), who observed a small but significant difference in TBARS content between frozen cod and haddock stored for up to 12 months at -10 and -30 °C. Dulavik *et al.* (1998) reported on higher contents of TBARS in dark muscle of saithe stored for nine months at -10 and -20 °C, compared to samples stored at -30 and -80 °C, though only a minor increase was observed in light muscle stored at -10 °C compared to the lower temperatures.

The change in TBARS for rainbow trout samples at different temperatures over time is illustrated in table 5, Paper II. The TBARS content increased with time for samples stored at -10 and -20 °C and was significantly higher compared to samples stored at or below -30 °C ( $p < 0.001$  for all) during the time of the experiment. For some reason the two fish analysed after one month of frozen storage generally had relatively high levels of TBARS at all temperatures compared to the 20 fish stored at 2-18 months. After excluding these two fish from the 1-way ANOVA, samples stored at -30 °C had significantly higher levels of TBARS ( $p < 0.001$  for all) than samples stored at lower temperatures. In tuna there was an obvious relationship between increased secondary lipid oxidation and increased storage temperature (table 6, Paper II). Samples stored at -10 °C had significantly higher contents of TBARS compared to samples stored at lower temperatures and samples stored at -20 and -40 °C higher than -50 to -80 °C. Samples stored at -30 °C had a significantly higher content of

TBARS compared to samples stored at -80 °C and there were no differences in content of TBARS for samples stored at -50 to -80 °C. Aubourg *et al.* (2004) observed higher peroxide value (PV) and TBARS content in horse mackerel fillets stored at -20 compared to -80 °C. Rodriguez *et al.* (2007) observed a relatively low degree of lipid oxidation (PV, TBARS) in farmed Coho salmon stored at -20 °C and did not observe any significant increase in TBARS content until after 15 months of frozen storage where the TBARS value was 0.54 mg/kg sample. This value corresponds approximately to the level of 8.3 µmol/kg sample for trout stored at -20 °C for eight months in our experiment, and the authors attributed the oxidative stability to stable endogenous antioxidants. The level of TBARS in our study did, however, not increase much from eight to 18 months. Trout samples stored at -10 and -20 °C in our experiment were placed directly in the freezer at these temperatures, therefore experiencing a slow freezing rate. According to Geromel and Montgomery (1980) a slow rate of freezing can cause lipase to be released from the lysosomes in rainbow trout and thereby enhance lipid oxidation, whereas intermediate or fast freezing rates do not result in significant increase in lipase release.

The rainbow trout experiment was set up to further check the results of the main experiment. The same five fish were stored at each of the three temperatures (-30, -40 and -50 °C) and times (two and six months) to minimize the effect of different individuals. 1 and 2-way ANOVA were used to analyse the data and the results of the experiment are illustrated in Figure 16. Samples stored at -30 °C had significantly higher levels of TBARS compared to samples stored at -40 ( $p < 0.01$ ) and -50 °C ( $p < 0.001$ ) and samples stored at -40 °C had significantly higher levels compared to samples stored at -50 ( $p < 0.01$ ). Moreover samples stored for six months had higher levels of TBARS than samples stored for two months ( $p < 0.0001$  for time in 2-way ANOVA). This verifies the results of the main experiment in which a significant difference between TBARS in samples stored at -30 compared to -40 and lower was shown for samples stored from 2-18 months. These results indicate that storage at -50 °C results in decreased lipid oxidation in rainbow trout fillets compared to storage at -30 or -40 °C after only 2-6 months of frozen storage. The reason for the increased sensibility towards oxidation of these rainbow trout compared to the ones used in the main experiment may be handling before slaughtering, bleeding or the fact that these trout were frozen in the pre-rigor state.

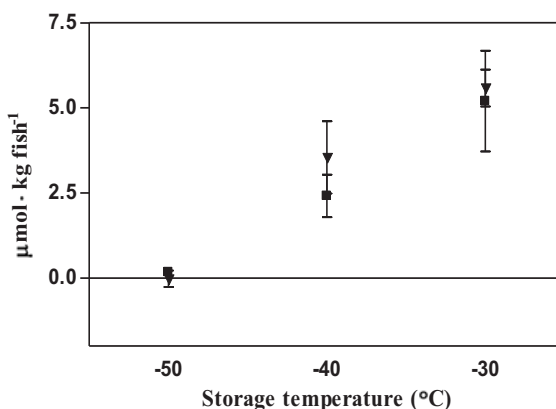


Figure 16. TBARS in frozen stored rainbow trout. Symbols used: ■, 3 months; ▲, 5 months. n=5.

All TBARS values are, however, below 8-10  $\mu\text{mol/kg}$  meat/fish which has been used as the detection limit of rancid off flavours in fresh pork and mackerel (Ke *et al.*, 1976; Lanari *et al.*, 1995). Sensory evaluations were not performed in this study, but a TBARS level of approximately 5  $\mu\text{mol/kg}$  fish would probably not result in quality deteriorating levels of rancid off flavours in samples stored at -30 °C for 2-6 months.

#### 7.2.6 Protein oxidation measured as carbonyl group determination

In Table 4 mean values for carbonyls in frozen stored cod are given. Samples stored at -10 °C had significantly higher levels of carbonyls (1- and 2-way ANOVA) than samples stored at all other temperatures and times indicating a higher degree of protein oxidation. When data for samples stored at -10 °C were excluded from the 1- and 2-way ANOVA, samples stored at -20 °C had significantly higher levels of carbonyls than samples stored at lower temperatures from approximately 10 months onwards. This is in agreement with Kjærsgård *et al.* and Baron *et al.* (2006a; 2007) who reported a significant increase in protein oxidation (carbonyl group determination) in rainbow trout fillets stored at -20 °C for 24 and 13 months, respectively, whereas no significant increase were observed in samples stored at -30 and -80 °C. Lipid oxidation is believed to induce protein oxidation and vice versa, though it is still unclear if the two processes occur concomitantly or if one precedes the other (Baron *et al.*, 2007). In this experiment no differences were observed in carbonyl levels in rainbow trout, though

increased levels of TBARS were seen in trout samples stored at -10 to -30 °C compared to lower temperatures. Moreover, increased carbonyl levels were observed in cod samples stored at -10 and -20 compared to lower, whereas no differences in TBARS levels were observed in cod.

The results for carbonyl group determination in rainbow trout did not show any effect of temperature ( $P > 0.05$ , results not shown).

**Table 4. Carbonyls (nmol carbonyl/mg protein) in cod fillets.**

Temp. (°C)		Frozen storage (months)																							
		1		2		3		4		5		6		8		10		12		15		18			
		Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
-10 <sup>b</sup>	1	3.42	0.16	2.90	0.04	3.53	0.79	2.46	0.06	8.54	2.00	8.54	2.00	2.98	0.46	7.79	1.37	7.76	0.58	11.48	1.79	16.83	0.1		
	2	4.57	1.04	4.59	0.49	3.02	0.39	3.64	2.07	3.99	0.39	3.99	0.39	4.40	0.30	8.18	0.32	9.29	0.90	12.60	1.31	13.34	1.2		
-20	1	1.94	0.46	1.67	0.11	0.76	0.66	0.77	0.17	2.51	0.43	2.51	0.43	1.12	0.16	1.62	0.62	3.47	2.05	3.67	0.28	3.64	0.5		
	2	1.05	0.27	1.75	0.22	1.00	0.43	1.15	0.35	1.13	0.61	1.13	0.61	1.63	0.17	3.31	0.32	3.12	0.79	2.20	0.23	4.92	0.1		
-30	1	1.15	0.18	1.59	0.21	0.63	0.30	1.04	0.56	2.13	0.56	2.13	0.56	0.40	0.07	1.36	0.30	1.67	0.39	1.28	0.11	1.31	0.2		
	2	1.36	0.28	1.48	0.21	0.64	0.17	0.84	0.74	1.01	0.16	1.01	0.16	0.80	0.54	0.44	0.15	1.10	0.37	1.38	0.08	1.35	0.4		
-40	1	1.19	0.69	1.46	0.23	1.08	0.35	1.32	0.12	0.96	0.01	0.96	0.01	0.58	0.08	0.80	0.25	0.60	0.12	0.85	0.22	1.40	0.3		
	2	1.49	0.47	1.99	0.20	0.73	0.27	0.58	0.32	1.74	1.14	1.74	1.14	0.60	0.01	0.77	0.06	0.67	0.25	1.25	0.36	1.25	0.1		
-50	1	1.38	0.05	2.18	0.50	0.56	0.03	0.54	0.16	1.14	0.69	1.14	0.69	0.62	0.06	0.61	0.05	0.41	0.21	0.70	0.11	1.14	0.0		
	2	1.08	0.06	1.38	0.10	0.84	0.30	0.67	0.09	2.08	0.13	2.08	0.13	0.56	0.03	0.73	0.29	0.73	0.19	0.93	0.18	1.03	0.0		
-60	1	1.23	0.09	1.15	0.25	0.57	0.13	0.46	0.10	0.29	0.23	0.29	0.23	0.69	0.06	0.53	0.02	0.64	0.07	0.72	0.07	1.22	0.3		
	2	1.27	0.17	1.47	0.14	0.31	0.31	0.65	0.55	1.72	1.03	1.72	1.03	0.87	0.18	0.42	0.13	1.00	0.24	1.12	0.07	1.00	0.0		
-70	1	1.11	0.37	1.28	0.03	0.63	0.09	0.66	0.28	0.97	0.09	0.97	0.09	0.89	0.26	0.55	0.05	0.89	0.29	1.05	0.06	1.29	0.1		
	2	1.04	0.10	1.71	0.52	0.71	0.11	0.62	0.12	1.37	0.31	1.37	0.31	0.88	0.11	0.52	0.10	1.44	0.22	1.22	0.38	1.41	0.3		
-80	1	1.16	0.15	1.44	0.29	0.82	0.17	0.40	0.09	0.94	0.12	0.94	0.12	0.78	0.14	0.68	0.13	0.66	0.22	1.43	0.31	1.44	0.3		
	2	0.75	0.08	1.45	0.13	1.15	0.28	0.53	0.09	1.06	0.18	1.06	0.18	0.90	0.10	0.60	0.12	0.77	0.52	1.52	0.14	1.21	0.2		
1-way ANOVA		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p = 0.017$		$p > 0.05$		$p = 0.001$		$p = 0.039$		$p = 0.001$			

a) n=3.

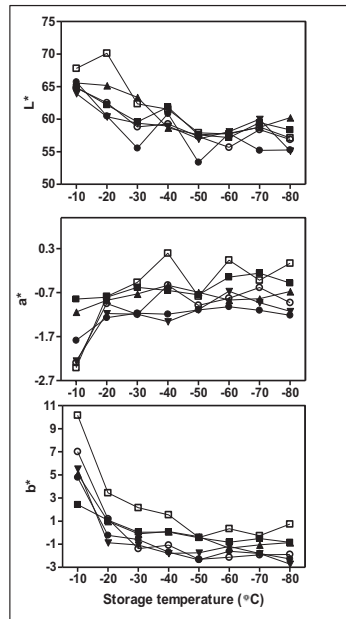
b) All  $p$ -values are for analyses without -10 °C.

2-way ANOVA: temperature,  $p = 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$

### 7.2.7 Colour

Colour measurements were performed using the CIE 1976,  $L^*$ ,  $a^*$ ,  $b^*$  colour space. Values for  $L^*$ ,  $a^*$  and  $b^*$  were analysed by 1-way ANOVA. The results of the colour measurements are shown in Figure 17. Cod samples stored at -10 and -20 °C were significantly lighter (higher  $L^*$ ) and yellowish (negative  $b^*$ ) than samples stored at lower temperatures. For samples stored at -10 °C there was a tendency towards higher  $b^*$ -values with increasing storage times. Moreover, samples stored at -10 °C had lower  $a^*$ -values than samples stored at

lower temperatures, indicating a more greenish colour. Similar results for changes in  $L^*$  - and  $b^*$  -values in frozen stored cod were obtained in a study by Schubring, who in one study (Schubring, 2005) observed increasing  $L^*$  - and  $b^*$  -values for samples stored at -20 and especially -14 °C compared to samples stored at -28 °C and in another (Schubring, 2004) increasing  $b^*$  - and  $L^*$  -values and decreasing a-values for -10 °C compared to -20 and -30 °C. General colour changes in cod include loss of surface glossiness, muscle opacity or chalky appearance and are probably due to irreversible changes in the muscle proteins (Shenouda, 1980).



**Figure 17. Colour ( $L^*$ ,  $a^*$ ,  $b^*$ ) of frozen stored cod samples.**  
 1-way ANOVA without -10 °C:  $L$ ,  $p < 0.0001$ ;  $a$ ,  $p > 0.05$ ;  $b$ ,  $p = 0.001$ .  
 Symbols used: ■, 5; ▲, 8; ▼, 10; ●, 12; ○, 15; □, 18 months.

Possible differences in colour due to storage temperature and time were blurred by large individual variations in the colour of the trout samples (results not shown).

Colour changes in tuna during storage are caused by the oxidation of the bright cherry red myoglobin to the brown metmyoglobin. In tuna, colour measurements were performed on intact as well as minced samples. The values for  $L^*$ ,  $a^*$  and  $b^*$  were analysed using 1- and 2-way ANOVA. In general, the  $a^*$ -values for whole and minced tuna fillets increased with

decreasing storage temperature (Test for linear trend  $p = 0.046$  (whole) and  $p < 0.0001$  (minced), Paper II, figure 3), while the  $L^*$ -values for whole and minced samples and the  $b^*$ -values for whole samples decreased with decreasing storage temperature (Test for linear trend  $p = 0.0125$  ( $L^*$ , whole),  $p = 0.0131$  ( $L^*$ , minced) and  $p = 0.03$  ( $b^*$ , whole)). The  $b^*$ -values for minced samples did not differ with storage temperature (Test for linear trend,  $p > 0.05$  for both). Tuna samples stored at  $-80\text{ }^{\circ}\text{C}$  were darker than samples stored at  $-10$  and  $-20\text{ }^{\circ}\text{C}$  and  $-60$  were darker than  $-10\text{ }^{\circ}\text{C}$  after three months of frozen storage (2-way ANOVA). After two months of frozen storage samples stored at  $-10$  and  $-20\text{ }^{\circ}\text{C}$  were significantly more brownish (lower  $a^*$ ) compared to samples stored at  $-40$  to  $-80\text{ }^{\circ}\text{C}$  and after three months also compared to samples stored at  $-30$  to  $-80\text{ }^{\circ}\text{C}$  (2-way ANOVA). This is in fair agreement with Watabe and Hashimoto (1986), who measured colour in tuna meat using the ratio of metmyoglobin to total myoglobin. They showed that this ratio was at the same level for tuna samples stored at  $-20$ ,  $-40$ ,  $-60$  and  $-80\text{ }^{\circ}\text{C}$  for the first three months of frozen storage, after which the ratio for samples stored at  $-20\text{ }^{\circ}\text{C}$  increased (became browner) until the end of the experiment (12 months) and samples stored at  $-40$  to  $-80\text{ }^{\circ}\text{C}$  remained unchanged. Chow *et al.* (1988) also demonstrated that discoloration of tuna meat proceeded steadily during storage at  $-20\text{ }^{\circ}\text{C}$  and was accelerated upon thawing, whereas storage below  $-30\text{ }^{\circ}\text{C}$  effectively prevented accelerated discoloration upon thawing.

### **7.2.8 Overview of reported quality-related changes in frozen fish**

In this section the quality-related changes occurring during frozen storage that have been referred to in the previous sections are collected in Table 5. The results obtained in the storage experiments in this project are collected in Table 6. Results for samples stored at  $-10\text{ }^{\circ}\text{C}$  are not reported in Table 6.

**Table 5. Reported quality-related changes in various fish species during frozen storage as result of differences in storage temperature.**

Species	Quality-related changes during frozen storage	Frozen storage (months)	Storage temperatures (°C)	Authors
Cod	Decreased WHC	10	-10/-20 vs. -30	Schubring, 2004 and 2005
Cod	Higher drip loss	10-11 days	-10/-20 vs. -40, -55, -70	Mørkøre and Lilleholt, 2007
Cod	More loosely bound water	10	-10 vs. -20/-70	Steen and Lambelet, 1997
Cod	More loosely bound water	> 3	-20 vs. -30	Jensen et al., 2003
Pacific whiting	Faster decrease in Ca <sup>2+</sup> -ATPase activity		-8 vs. -20, -34, -50	Hsu et al., 1993
Cod	Lower Ca <sup>2+</sup> -ATPase activity		-20 vs. -30	Godiksen et al., 2003
Hake	No difference in Ca <sup>2+</sup> -ATPase activity	11	-20 and -30	del Mazo et al., 1999
Trout	Increased lysosomal leakage and higher enzyme activity		-18 vs. -40	Nilsson and Ekstrand, 1995
Cod, haddock	Increased TBARS content	12	-10 vs. -30	Aubourg and Medina, 1999
Saithe, dark muscle	Increased TBARS content		-10/-20 vs. -30/-80	Dulavik et al., 1998
Saithe, light muscle	Minor increase in TBARS content		-10 vs. -20, -30, -80	Dulavik et al., 1998
Horse mackerel	Higher PV and TBARS		-20 vs. -80	Aubourg et al., 2004
Cod	Increase in L*- and b*-values		-14/-20 vs. -28	Schubring, 2004
Cod	Increase in L*- and b*-values, decrease in a*-values		-10 vs. -20/-30	Schubring, 2004
Tuna	No colour difference	0-3	-20, -40, -60, -80	Watabe and Hashimoto, 1986
Tuna	More brownish colour	3-12	-20 vs. -40, -60, -80	Watabe and Hashimoto, 1986
Tuna	More brownish colour		-20 vs. -30	Chow et al., 1988
Cod	Higher levels of carbonyls	13	-20 vs. -30 and -80	Baron et al, 2007
Cod	Higher levels of carbonyls	24	-20 vs. -30 and -80	Kjærsgård et al, 2006a



**Table 6. Collection of the results of the storage experiments of this project. The effect of temperature on various quality-related changes during frozen storage. Results for samples stored at -10 are not reported in the table.**

Species	Quality-related changes during frozen storage	Frozen storage (months)	Storage temperatures (°C)
Cod	Higher drip loss	1-18	-20 vs. -30 to -80
Cod	Decreased WHC	5-18	-20 vs. -30 to -80
Rainbow trout	Higher drip loss	1-18	-20 vs. -30 to -80
Rainbow trout	Decreased WHC	12-18	-20 vs. -30 to -80
Tuna	WHC	2-3	No diff. between -20 to -80
Cod	Changed water distribution	4/5-18	-20 vs. -30 to -80
Cod	Changed water distribution	12-18	-30 vs. -40 to -80
Rainbow trout	Changed water distribution	12-18	-20 vs. -30 to -80
Cod	Lower Ca <sup>2+</sup> -ATPase activity	1-18	-20 vs. -40 to -80
Rainbow trout	Lower Ca <sup>2+</sup> -ATPase activity	1-18	-20 vs. -30 to -80
Cod	Lower cathepsin D activity	1-18	-20 vs. -30 to -80
Cod	No differences in TBARS content	1-18	-20 vs. -30 to -80
Rainbow trout	Higher and increasing TBARS content	1-18	-20 vs. -30 to -80
Rainbow trout	Higher TBARS content	2-18	-30 vs. -40 to -80
Tuna	Higher TBARS content	2-3	-20 and -40 vs. -50 to -80
Tuna	Higher TBARS content	2-3	-30 vs. -80
Rainbow trout	Higher and increasing TBARS content	3-5	-30 vs. -40 and -50
Rainbow trout	Higher and increasing TBARS content	3-5	-40 vs. -50
Cod	Higher levels of carbonyls	10-18	-20 vs. -30 to -80
Cod	More light and yellowish	5-18	-20 vs. -30 to -80
Tuna	More brownish colour	2/3	-20 vs. -40/-30 to -80

### 7.3 DSC experiments

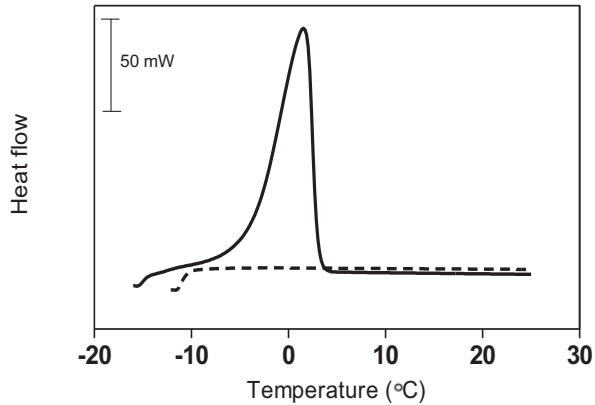
Four DSC experiments were carried out and as these were named experiment 1, 2, 3 and 4 in Paper III this will also be the case in this section. An overview is given in Table 7.

**Table 7. Scanning rate, annealing temperature and annealing time in the DSC-experiments.**

Exp.	Samples	Scanning rate (°C/min)	Annealing time (min)	Annealing temperature (°C)
1	Fresh tuna	10	30	-10, -25, -35, -47, -60, -80
2	Pre-frozen cod	10	20, 30, 60, 120, 180, 240	-20, -60
3	Pre-frozen tuna and herring, fresh and pre-frozen cod	1 or 10	10 or 30	-10, -12, -14, -16, -18, -20, -22
4	Fresh and pre-frozen cod	1 or 10	Up to eight hours	-5, -8, -10, -12, -14, -16, -20

DSC experiment 1 were carried out to check if annealing at different temperatures between -10 and -80 °C for various times would result in any differences that could be seen from the thermograms obtained when scanning the samples.

In experiment 1, six annealing temperatures (-10, -25, -35, -47, -60 and -80 °C) were chosen to see if the amount of unfrozen water decreases with decreasing temperature. The specific enthalpy changes (per g muscle) for ice melting during the subsequent heating are listed in table 2, Paper III. Annealing for 30 min at -10 °C followed by heating to 20 °C did not result in a melting curve; thus, no ice crystallisation had occurred. With annealing temperatures between -25 and -80 °C, no significant difference in amount of unfrozen water was found ( $p>0.05$ ). Figure 18 illustrates thermograms for cod samples annealed at -12 (no ice crystallisation) and -16 °C (ice crystallisation resulting in a melting peak upon heating).



**Figure 18.** Example of DSC thermograms (heating curves) for two cod samples cooled to and annealed at  $-16^{\circ}\text{C}$  (solid line) and  $-12^{\circ}\text{C}$  (dashed line), respectively. The scanning rate was  $1^{\circ}\text{C}/\text{min}$ . Endothermic heat flow is shown in the positive direction. From Paper III.

Experiment 2 was set up to verify the results from experiment 1 and to check if the annealing time (20 to 240 min) had any influence on the amount of unfrozen water at  $-20$  or  $-60^{\circ}\text{C}$  (Table 8). No significant temperature dependence was found (*t*-test), nor was a time dependence (linear regression) at any of the two temperatures. In conclusion, the amount of unfrozen water in the muscle does not correlate with annealing time or temperature, but ice formation seems to be an abrupt phenomenon: when passing below the ice crystallisation point, some of the water turns into ice and a constant fraction remains unfrozen, probably in a trapped non-equilibrium state.

**Table 8. Unfrozen water in cod muscle after 20 to 240 min annealing at -20 and -60 °C. The scanning rate was 10 °C/min. From Paper III.**

Annealing temperature (°C)	Annealing time (min)	Unfrozen water (%)
-20	20	26.9
	30	29.1
	60	25.3
	120	24.0
	180	27.7
	240	28.0
-60	20	27.3
	30	25.1
	60	24.6
	120	28.8
	180	26.6
	240	28.3

Table 9 shows the results of experiment 3. All water in fresh and pre-frozen cod muscle remained unfrozen down to -14 to -16 °C, slightly depending on the cooling rate. At lower temperatures, pre-frozen cod had a small, but significant ( $p<0.001$ ) decrease in the fraction of unfrozen water (18.9 %) compared to fresh cod (23.7 %). In pre-frozen herring muscle, the ice crystallisation point was around -12 to -14 °C, again depending on the scanning rate. Only 14.2 % of the water in these samples remained unfrozen at the lower temperatures. Prefrozen tuna showed a similar ice crystallisation point except for one sample in which ice formation took place at -12 and -10 °C also (data not included).

**Table 9. Percent unfrozen water (of total water content) in fish muscle at different annealing temperatures. The annealing time was 10 min. From Paper III.**

Temperature (°C)	Scanning rate (°C/min)					
	Fresh cod		Prefrozen cod		Prefrozen herring	
	1	10	1	10	1	10
-22	24.1	22.0	17.2	20.4	-	-
-20	24.1	24.6	18.4	20.5	13.1	12.7
-18	23.7	25.1	19.0	17.5	16.3	8.4
-16	23.3	100.0	19.6	100.0	16.7	15.5
-14	100.0	100.0	100.0	100.0	12.0	18.8
-12	-	-	100.0	100.0	100.0	100.0
-10	-	-		-	100.0	100.0

In experiment 4, different combinations of annealing temperature and time were tried in an attempt to incite ice crystallisation at temperatures higher than -14 °C under various conditions. Table 10 shows the results of the experiment. Slow cooling (1 °C/min) to -12 °C or -14 °C in steps of 2 °C with annealing for 2 min at each step during the cooling did not incite crystallisation and the cod muscle remained unfrozen after 10 min annealing at the two temperatures. Increasing the annealing time to 8 hours did not result in freezing of samples at -10 °C. At -14 °C one sample froze after two hours whereas another did not freeze even during four hours of annealing.

The main result of the DSC experiments was that no significant dependence of temperature on the amount of unfrozen water existed even down to -80 °C. Thus all freezable water was frozen at -20 °C or even higher. This is opposite to an earlier use of -40 °C (Chen, 1985a; Chen, 1985b; Pham, 1987; Aktas *et al.*, 1997a; Aktas *et al.*, 1997b; Boonsupthip and Heldman, 2007) or -70 °C (Simatos *et al.*, 1975; Ross, 1978) as limits at which all freezable water is frozen. It should, however, be noted that if the unfrozen water fraction cools down very fast, the viscosity becomes so high that water diffusion is very slow and thereby also ice crystallisation or crystal growth takes very long time (Franks, 1986; Wolfe *et al.*, 2002). Therefore these results might look different if it had been possible to use another time scale.

**Table 10. Lack of ice crystallisation at various combinations of annealing temperature and time. The scanning rate was 1 °C/min. From paper III.**

Annealing temperature (°C)	Annealing time (min)	Ice crystallisation
-12	2 min at respectively -2, -4, -6, -8 and -10 °C and 10 min at -12 °C	None
-14	2 min at respectively -4, -6, -8, -10 and -12 °C and 10 min at -14 °C	None
-10	60	None
-10	10	None
-10	480	None
-12	10	None
-14	10	None
-14	240	None
-14	480	Froze after 120 min

The average amount of unfrozen water in cod muscle found in experiment 2 was similar to values reported by Aktas *et al.* (1997b), Duckworth (1971) and Sablani *et al.* (2007). Aktas *et al.* (1997b) reported the amount of unfrozen water in beef at -5 and -10 °C to be 49 and 44 %, respectively. Between -15 and -40 °C the average amount of unfrozen water was at a stable level at 31 % and at -50 and -65 °C the amount of unfrozen water was reported to 29 %. They used the same principle (Roos, 1986) for calculating the amount of unfrozen water as in this study and concluded that the unfrozen water content reached a stable level between -15 and -40 °C , though dropping further until -50 to -65 °C. In our opinion, there is no evidence in the paper for a difference in the reported values for unfrozen water between -15 and -65 °C and therefore the results are in agreement with ours. Duckworth (1971) used differential thermal analysis (DTA) and determined the amount of unfrozen water to be 26 to 27 % in cod muscle. Sablani *et al.* (2007) used a method based on  $\Delta H$ -values for ice melting as a function of water content as well as a model based on the Chen (1986) equation and freezing curve data . They reported the unfrozen water content in king fish muscle to be 37 and 31 %, depending on the method used. Riedel (1956) and Charm and Moody (1966), both using calorimetric methods, reported much lower values for the unfrozen water content: 9 % at -40 °C in cod and 11 % in lean beef and haddock muscle. Bartlett (1944), Chen (1985b) and Pham (1987) calculated values between 2 and 8 % unfrozen or bound water at -40 °C in cod

and between 2 and 11 % in lean beef. The calculations were based on mathematical models, and both Chen (1985b) and Pham (1987) based the calculations on Riedel's (Riedel, 1956; 1957) experimental values. According to Pham (1987) the calculated differences between percentage of 'bound' and unfrozen water differed approximately 3 %. These calculations were based on the assumption of equilibrium between frozen and unfrozen water. As mentioned earlier, unfrozen water located between membranes or macromolecules should be able to remain unfrozen even in the presence of ice crystals because solutes and low temperatures can increase the viscosity to an extent where equilibrium is unreachable during a normal time period. Therefore the amount of experimentally determined unfrozen water is usually higher than the calculated values (Wolfe *et al.*, 2002). Still; this does not explain the difference between our results and the older results of Riedel (1956) and Charm and Moody (1966).

The relatively long annealing times in experiment 2 were used to ensure enough time for ice crystallisation. As mentioned before, it turned out that the annealing times did not affect the amount of unfrozen water. Therefore, annealing times of 10 min were used for the following DSC-measurements. These focussed on the existence of super-cooled water in cod and tuna muscle. The water in fresh as well as pre-frozen cod muscle remained unfrozen down temperatures lower than -14 °C. Herring muscle water remained unfrozen down to -12 °C and the water in tuna muscle was unfrozen at -10 °C when a fast cooling rate (10 °C/min) was used. For cod and herring there seemed to be a correlation between cooling rate and degree of super-cooling; this was also observed by Rahman and Driscoll (1994) and Rahman *et al.* (2003). Rahman *et al.* (2003) concluded, however, that for cooling rates at or below 1.5 °C/min, no significant differences in ice crystallisation points were observed. Moreover, their results showed only a minor degree of super-cooling in tuna. In order to insure that the observed super-cooling was not a result of a too high cooling rate, it was tried to incite ice crystallisation using slow cooling rates and annealing at different temperatures during cooling to the final annealing temperature. This did not result in ice crystallisation at -10 or -12 °C, and at -14 °C it took two hours before the water in the cod sample (approximately 15 µg) froze. This means that the cod muscle is in a super-cooled, non-equilibrium state. Aktas *et al.* (1997b) when cooling meat samples to -5 or -10 °C at 5 °C/min in a DSC followed by heating to 35 °C, reported that all samples froze thus no noticeable super-cooling was observed. This finding is in disagreement with our results, though the methods used are comparable.

However, Aktas *et al.* (1997b) used minced beef meat which may contain more loosely bound water with other thermodynamic properties than the whole fish samples used in our experiment as is also reported to be the case for minced cod compared to whole cod (Jensen *et al.*, 2002). When DSC-samples are cooled to, say -20 °C, the extent of super-cooling is difficult to determine because the position of the resulting freezing exotherm depends on several factors like the cooling rate and the thermal conductivity. The melting peak obtained during a successive heating is commonly used to estimate the equilibrium freezing/melting temperature. With this approach, the heating curve is also used for indirect measurement of the ice crystallisation point by finding a narrow temperature interval within which the melting peak disappears. Very small volumes of pure solute can super-cool tens of degrees Celsius, but biological solutions, such as fish muscle, usually contain ice nucleators that initiate ice crystallisation when cooled to a few degrees Celsius below zero (Wolfe and Bryant, 2001). Fast cooling rates result in a rapidly rising viscosity, which might decrease the probability of ice nucleation due to low molecular mobility. This may partly explain the difference observed between ice crystallisation temperatures for fish cooled at rates of 1 and 10 °C/min. A cod sample cooled to -10 °C was kept for 8 hours without freezing. For practical reasons, longer experimental times were not used, so the maximum time the cod can remain in this super-cooled, non-equilibrium state is not known. Also, the super-cooling of fish muscle to the extent described here may differ in samples of a larger scale as the probability of nucleation increases with the volume of the sample in super-cooled liquids (Wolfe and Bryant, 2001) and moreover the high degree of super-cooling may occur in the DSC pan only.

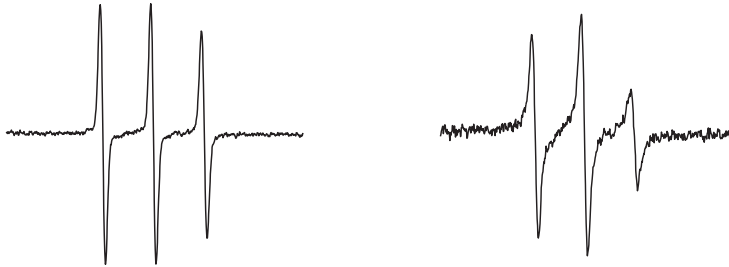


## 7.4 ESR experiment

Electron spin resonance can be used to follow the mobility of a spin probe added to a muscle sample during freezing. This can give information about the viscosity in the unfrozen solution during freezing, which alters due to ice crystallisation (concentration of the unfrozen water fraction) but also due to changes in temperature.

Different combinations of time constant, conversion time, receiver gain and number of accumulated scans were tried to find the optimal settings with regard to noise and time consumption.

The first scan was run at 0 °C to obtain an isotropic nitroxyl spectrum (illustrated in Figure 19). A gradual lowering of the temperature made the spectra noisier due to decreasing mobility of the added spin probe.



**Figure 19.** ESR spectra of cod treated with the nitroxyl spin probe TEMPOL recorded at 0 °C (left) and -1.7 °C (right). Receiver gain:  $2.5 \cdot 10^5$ , conversion time: 81.92 ms and time constant 81.92 ms.

A number of different combinations of receiver gain (RG), conversion time (CT) and time constant (TC) were tried to optimize the appearance of the spectra at different temperatures. When time consumption as well as degree of noise were considered the following settings were found to be optimal: RG:  $3.2 \cdot 10^5$ , CT: 327.68 ms, TC 327.68 ms and number of scans: 1. Figure 20 shows an ESR spectrum recorded on cod at -9 °C using these settings.



**Figure 20.** ESR spectrum of cod treated with the nitroxyl spin probe TEMPOL recorded at -9 °C. Receiver gain:  $3.2 \times 10^5$ , conversion time: 327.68 ms and time constant 327.68 ms.

These settings were used when recording ESR spectra of the TEMPOL treated cod sample at temperatures ranging from -5.4 to -40.2 °C. Unhindered mobility of the added probe results in an isotropic nitroxyl spectrum (see Figure 19 left) whereas a reduced mobility of the added spin probe caused by lowering of the temperature results in a nitroxyl powder spectrum.

Figure 21 shows the development from an isotropic nitroxyl spectrum (-5.4 °C) to a typical nitroxyl powder spectrum (-21.0 °C and lower). At -5.4 °C the rotational mobility of the spin probe is already hindered to some degree as can be seen from the development of a little bump on the first line and the decreasing height of the third line compared to the middle one. At -21.0 °C the rotational mobility of the spin probe is hindered because the crystallisation of water gives rise to increased viscosity of the concentrated liquid phase.



**Figure 21.** ESR spectra of cod treated with the nitroxyl spin probe TEMPOL recorded at temperatures from -5.4 °C to -40.2 °C. Receiver gain:  $3.2 \times 10^5$ , conversion time: 327.68 ms and time constant 327.68 ms.

The changes of the spectra caused by decreasing rotational mobility of the spin probe can be quantified using the same method as Hansen *et al.* (2004) and Orlén *et al.* (2004). The height of the low field peak from the powder spectrum ( $h_p$ , see Figure 22) and the height of the peak of the high field isotropic spectrum ( $h_i$ ) were normalized relative to the height of the central peak ( $h_c$ ). For a typical isotropic spectrum the ratio  $h_i/h_c$  are near 1.

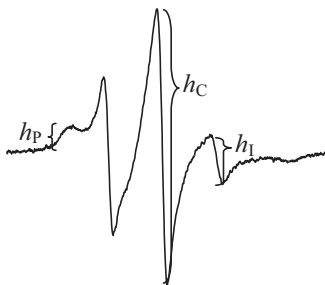


Figure 22. Determination of the peak heights  $h_P$ , the height of the low field peak,  $h_I$ , the height of the peak of the high field isotropic spectrum and  $h_C$ , the height of the central peak.

The temperature dependence of the peak height ratios of the spectra in Figure 22 are illustrated in Figure 23.

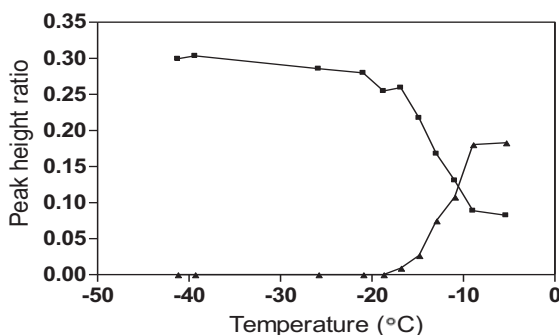
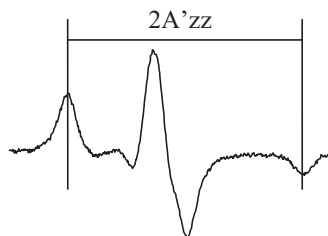


Figure 23. Spectral changes of the ESR spectra for the nitroxyl spin probe TEMPOL in cod measured as the relative height of the powder peak,  $h_P/h_C$  (■) and the isotropic peak,  $h_I/h_C$  (▲).

At temperatures below  $-21$  °C the relative height of the powder peak,  $h_P/h_C$ , is constant and the isotropic peak has disappeared. From  $-21$  °C to  $-9$  °C the relative height of the powder peak and the isotropic peak, respectively, decreases and increases before levelling off between  $-9$  and  $-5$  °C. Unfortunately it was difficult to adjust the ESR spectrometer at temperatures above  $-5$  °C, because water absorbs microwaves very efficiently. Therefore no spectra were measured between  $-5$  and  $0$  °C on this cod sample. The relative height of the isotropic peak in this interval is unknown, but the relative height at  $0$  °C must be assumed to be near 1 and the value at  $-5$  °C therefore seems to be very low. The changes in the shapes of the spectra above  $-21$  °C are caused by the melting of ice which results in a decreasing viscosity of the liquid phase. These results agree with ESR spectra recorded on tuna, where a change toward an

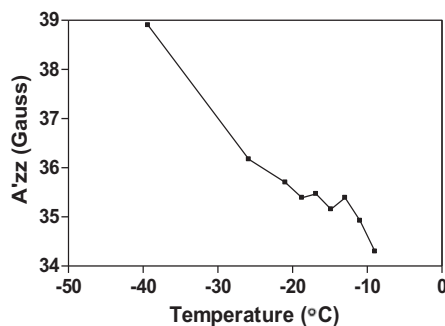
isotropic three-line spectrum begins at  $-25\text{ }^{\circ}\text{C}$  (Orlien *et al.*, 2004) In TEMPOL treated pork (Hansen *et al.*, 2004) the shapes of the ESR spectra change dramatically above  $-40\text{ }^{\circ}\text{C}$  indicating initial ice melting. This discrepancy can, however, be due to differences between cod and pork.

The hyperfine coupling constant,  $A'_{zz}$ , is determined as half of the splitting between the outer two peaks of the powder spectrum (see Figure 24) and can be used to quantify the temperature dependent changes in the powder spectrum (Dzuba, 1996; Hansen *et al.*, 2002).



**Figure 24.** The hyperfine coupling constant,  $A'_{zz}$ , was determined as the half of the splitting between the outer two peaks of the powder spectra.

Figure 25 shows a plot of the hyperfine coupling constant,  $A'_{zz}$ , as a function of temperature.  $A'_{zz}$  increases with decreasing temperature, as the rotational mobility of the spin probe decreases. This is in agreement with Hansen *et al.* (2004), who obtained similar results in pork in the temperature interval between  $-55$  to  $-10\text{ }^{\circ}\text{C}$ .



**Figure 25.** The hyperfine coupling constant,  $A'_{zz}$  plotted as a function of temperature for TEMPOL treated cod.

The results of the ESR measurements partly seem to support the results of the DSC measurements as the isotropic peak disappears completely below  $-21\text{ }^{\circ}\text{C}$  indicating hindrance of the rotational mobility of the spin probe. It may be that all the 'freezable' water has crystallised, thereby resulting in no further concentration of the unfrozen water phase. It is, however, difficult to interpret the ESR spectra when it comes to the exact beginning and ending of ice crystallisation because ice crystallisation as well as a lowering of the temperature both result in increased viscosity and thereby hindered mobility of the spin probe. Another issue making it difficult to compare the results of the ESR and DSC measurements is that it was almost impossible to tune the Bruker spectrometer before recording of a spectrum without using a low temperature (e.g.  $-20\text{ }^{\circ}\text{C}$ ). Therefore the sample was already frozen at a lower temperature when it was measured at e.g.  $-10\text{ }^{\circ}\text{C}$ . In contrast to this, samples annealed at  $-10\text{ }^{\circ}\text{C}$  in the DSC were in a super-cooled unfrozen state.

## 8 Conclusion

The primary objective of this work was to investigate the relation between quality-related changes in frozen fish and storage temperatures in the interval from -20 to -80 °C.

Temperature intervals of 10 degrees Celsius were used and the experiments were all paired with regard to temperature. This is in contrast to most other studies that are paired with regard to time. The combination of using eight different storage temperatures, a temperature-paired design and a variety of analyses gave an opportunity to study quality-related changes in frozen fish in a broad temperature interval. Combined with the use of multivariate data analysis, possible relations between changes in quality-related characteristics, storage temperature and time could be observed, thereby giving the opportunity to change the experimental design and focus on a narrower temperature interval.

Moreover, the importance of freezing and thawing rates were investigated in pre- and post-rigor cod. Ice crystallisation point and amount of unfrozen water in fish muscle during freezing were also investigated using DSC, and the mobility of an added spin probe in the unfrozen water fraction in frozen cod muscle was measured using ESR.

The main conclusions of my PhD-project are as follows:

- A storage temperature of -30 °C was sufficiently low for the maintenance of a high quality of post-rigor cod for 12 months of frozen storage. In general, storage at -30 or lower compared to -20 °C influenced the quality-related changes positively in terms of drip loss, WHC,  $\text{Ca}^{2+}$ -ATPase and cathepsin D activity, carbonyl content and colour, whereas the level of TBARS was not influenced. The results of the NMR measurements do, however, indicate that long time storage (more than 12 months) at -40 °C or lower compared to -30 °C or higher results in less pronounced changes in water holding capacity and water distribution and therefore a storage temperature of -40 °C is advisable for the storage of cod for more than 12 months if the aim is to preserve the quality in the best way.
- Pre-rigor cod was more susceptible to handling procedures such as the rate of freezing and thawing than post-rigor cod, it do however seem as if many factors affect the amount of drip loss in pre-rigor cod.
- For rainbow trout -30 °C was sufficiently low for the prevention of significant quality deteriorating processes during long-term frozen storage. Storage at -40 °C and lower,

did however, seem to reduce the level of secondary lipid oxidation (TBARS) compared to storage at higher temperatures, but after 18 months of frozen storage at -30 °C, the value for TBARS was still below the sensory detection limit. No changes in quality-related parameters such as drip loss, WHC, water distribution and membrane stability (Ca<sup>2+</sup>ATPase and cathepsin D activity) were observed at -20 °C compared to lower temperatures.

- In tuna stored up to 3 months only changes in secondary lipid oxidation (TBARS) and colour were observed, and storage at -30 °C seemed to prevent discoloration (change to brownish colour) and lipid oxidation better than storage at -20 °C.
- During freezing of cod, herring and tuna in a DSC a super-cooling of more than 10 degrees Celsius occurred. Moreover, a relatively large amount of the muscle water remained unfrozen during freezing down to -80 °C and all 'freezable' water was frozen at -20 °C or even higher. The mobility of an added spin probe decreased at temperatures down to -21 °C and thereafter levelled off which could indicate complete ice crystallisation at this point in accordance with the DSC-measurements.

In conclusion, a storage temperature of -40 for long-term and -30 °C for short-term storage of cod, of -40 °C for rainbow trout and of -30 °C for tuna (up to 3 months) is advisable, whereas lower storage temperatures are not recommended from an economic and environmental point of view.

Since the thawing experiments with especially pre-rigor fish gave very diverse results, future experiments with different thawing methods would contribute with more knowledge regarding more optimal thawing procedure for pre-rigor fish. This is of high importance because a large number of fish are frozen at sea in the pre-rigor state.

The very large degree of super-cooling observed in the DSC experiment is probably not of importance for the industrial freezing of fish, but in relation to freezing of fish in domestic or supermarket freezers it may constitute a problem. Those freezers may not be sufficiently cold (-18 °C) and the actual ice crystallisation temperature of the fish may be so low that freezing has not yet occurred at, say, -15 °C. Therefore it could be of interest to study the ice crystallisation in fish further, using combinations of DSC, ESR and NMR and also to study the phenomenon in larger scale using a calorimeter.



## 9 References

- Agustini, T.W., Suzuki, T., Hagiwara, T., Ishizaki, S., Tanaka, M., and Takai, R. (2001). *Fisheries Science*, 67(2): 306-313.
- Aktas, N., Tulek, Y., and Gokalp, H.Y. (1997a). *Journal of Thermal Analysis*, 50(4): 617-624.
- Aktas, N., Tulek, Y., and Gokalp, H.Y. (1997b). *Journal of Thermal Analysis*, 48(2): 259-266.
- Alizadeh, E., Chapleau, N., and LeBail, A. (2007). *Innovative Food Science and emerging Technologies*, 8: 493-499.
- Almandos, M.E., Giannini, D.H., Ciarlo, A.S., and Boeri, R.L. (1984). *Lebensmittel-Wissenschaft und -Technologie*, 17(2): 111-113.
- Andersen, C.M. and Jørgensen, B.M. (2004). *Journal of Aquatic Food Product Technology*, 13(1): 13-23.
- AOCS (1998). Official Method Ce 1 b-89. Fatty Acid Composition by GLC. Marine Oils. AOCS, Champaign, IL.
- AOCS (1998). Official Method Ce 2-66. Preparation of methyl esters of fatty acids. AOCS, Champaign, IL.
- Andersen, C.M. and Rinnan, A. (2002). *Lebensmittel-Wissenschaft und -Technologie*, 35(8): 687-696.
- Aubourg, S. and Medina, I. (1997). *Journal of Agricultural and Food Chemistry*, 45(9): 3617-3621.
- Aubourg, S.P. (1999). *Food Research International*, 32(7): 497-502.
- Aubourg, S.P. and Medina, I. (1999). *Journal of the Science of Food and Agriculture*, 79(13): 1943-1948.
- Aubourg, S.P., Pineiro, C., and Gonzalez, M.J. (2004). *Journal of the American Oil Chemists' Society*, 81(7): 671-678.
- Babbitt, J.K., Crawford, D.L., and Law, D.K. (1972). *Journal of Agricultural and Food Chemistry*, 20(5): 1052-1054.
- Badii, F. and Howell, N.K. (2002a). *Journal of the Science of Food and Agriculture*, 82(1): 87-97.

- Badii, F. and Howell, N.K. (2002b). *Food Hydrocolloids*, 16(4): 313-319.
- Baron, C.P., Kjaersgard, I.V.H., Jessen, F., and Jacobsen, C. (2007). *Journal of Agricultural and Food Chemistry*: 55 (20) 8118-8125, 55(20): 8118-8125.
- Barroso, M., Careche, M., and Borderias, A.J. (1998). *Trends in Food Science & Technology*, 9(6): 223-229.
- Bartlett, L.H. (1944). *Refrigeration Engineering*, 47: 377.
- Bertram, H.C., Karlsson, A.H., Rasmussen, M., Pedersen, O.D., Donstrup, S., and Andersen, H.J. (2001). *Journal of Agricultural and Food Chemistry*, 49(6): 3092-3100.
- Bevilacqua, A., Zaritzky, N.E., and Calvelo, A. (1979). *Journal of Food Technology*, 14(3): 237-251.
- Bligh, E.G. and Dyer, W.J. (1959). *Canadian Journal of Biochemistry and Physiology*, 37: 911-917.
- Boonsupthip, W. and Heldman, D.R. (2007). *Journal of Food Science*, 72(5): E254-E263.
- Brown, W.D. 1986. Fish muscle as food. In: Muscle as food. Bechtel, P. J. (Ed.) Academic Press Inc., Orlando. pp. 405-451.
- Buttkus, H. (1970). *Journal of Food Science*, 35: 558-562.
- Cappeln, G., Nielsen, J., and Jessen, F. (1999). *Journal of the Science of Food and Agriculture*, 79(8): 1099-1104.
- Careche, M., del Mazo, M.L., Torrejon, P., and Tejada, M. (1998). *Journal of Agricultural and Food Chemistry*, 46(4): 1539-1546.
- Careche, M., Herrero, A.M., Rodriguez-Casado, A., del Mazo, M.L., and Carmona, P. (1999). *Journal of Agricultural and Food Chemistry*, 47(3): 952-959.
- Castell, C.H., Neal, W., and Smith, B. (1970). *Journal of the Fisheries Research Board of Canada*, 15: 701-716.
- Castell, C.H., Smith, B., and Dyer, W.J. (1973). *Journal of the Fisheries Research Board of Canada*, 30: 1205-1213.
- Chapman, K.W., Ifat, S., Keum Taek, H., and Regenstein, J.M. (1993). *Journal of Food Science*, 58(6): 1208-1211.

- Charm, S.E. and Moody, P. (1966). *Ashrae Journal*, 39-42.
- Chen, C.S. (1985a). *Journal of Food Science*, 50(4): 1158-1162.
- Chen, C.S. (1985b). *Journal of Food Science*, 50(4): 1163-1166.
- Chen, C.S. (1986). 51(6): 1537-1539.
- Chen, Y.-L. and Pan, B.S. (1997). *International Journal of Food Science & Technology*, 32(2): 159-168.
- Chow, C.J., Ochiai, Y., Watabe, S., and Hashimoto, K. (1988). *Bulletin of the Japanese Society of Scientific Fisheries*, 54(4): 639-648.
- Christian, J.H.B. and Scott, W.J. (1953). *Australian Journal of Biological Sciences*, 6(4): 565-573.
- Davies, K.E. and Nowak, K.J. (2006). *Nature Reviews Molecular Cell Biology*, 7(10): 762-773.
- del Mazo, M.L., Torrejon, P., Careche, M., and Tejada, M. (1999). *Journal of Agricultural and Food Chemistry*, 47(4): 1372-1377.
- Dingle, J.R., Keith, R.A., and Lall, B. (1977). *Canadian Institute of Food Science and Technology Journal*, 10(3): 143-146.
- Duckworth, R.B. (1971). *Journal of Food Technology*, 6: 317-327.
- Dulavik, B., Sorensen, N.K., Barstad, H., Horvli, O., and Olsen, R.L. (1998). *Journal of Food Lipids*, 5(3): 233-245.
- Eide, O., Børresen, T., and Strøm, T. (1982). *Journal of Food Science*, 47: 347-349, 354.
- Erikson, U., Beyer, A.R., and Sigholt, T. (1997). *Journal of Food Science*, 62(1): 43-47.
- Fennema, O. 1973. Nature of the freezing process. In: Low temperature preservation of foods and living matter. Fennema, O., Powrie, W. D. and Marth, E. H. (Ed.) Marcel Dekker, New York. pp. 151-239.
- Fernandez, P.P., Otero, L., Martino, M.M., Molina-Garcia, A.D., and Sanz, P.D. (2008). *European Food Research and Technology*, 227(5): 1367-1377.
- Fikiin, K.A. (1998). *Journal of Food Engineering*, 38: 331-339.

- Franks, F. (1982). *Cereal Foods World*, 27(9): 403-407.
- Franks, F. (1986). *Cryoletters*, 7: 207.
- Geromel, E.J. and Montgomery, M.W. (1980). *Journal of Food Science*, 45(3): 412.
- Gill, T.A., Keith, R.A., and Lall, B.S. (1979). *Journal of Food Science*, 44(3): 661-667.
- Godiksen, H., Hyldig, G., and Jessen, F. (2003). *Journal of Food Science*, 68(8): 2579-2585.
- Godiksen, H. and Jessen, F. (2001). *Journal of Food Biochemistry*, 25(4): 343-358.
- Han, T.J. and Liston, J. (1987). *Journal of Food Science*, 52(2): 294-&.
- Hansen, E., Andersen, M.L., and Skibsted, L.H. (2003). *Meat Science*, 63(1): 63-67.
- Hansen, E., Lauridsen, L., Skibsted, L.H., Moawad, R.K., and Andersen, M.L. (2004). *Meat Science*, 68(2): 185-191.
- Herrero, A.M., Carmona, P., and Careche, M. (2004). *Journal of Agricultural and Food Chemistry*, 52(8): 2147-2153.
- Howell, N., Shavila, Y., Grootveld, M., and Williams, S. (1996). *Journal of the Science of Food and Agriculture*, 72(1): 49-56.
- Hsu, C.K., Kolbe, E., Morrissey, M.T., and Chung, Y.C. (1993). *Journal of Food Science*, 58(5): 1055-1056.
- Hultin, H.O. 1992. Advances in seafood chemistry, composition and quality. In: Fleck, G. J. and Martin, R. E. (Ed.) Technomic Publishing, Lancaster, PA. pp. 25
- Inoue, N., Takatori, K., Motoshige, T., and Shinano, H. (1992). *Bulletin of the Japanese Society of Scientific Fisheries*, 58(12): 2357-2360.
- Isengard, H.D., Labuza, T.P., Lillford P.J., and Reid D S .(2008). *IUFoST Scientific Information Bulletin*, 1-10.
- James, C., Lejay, I., Tortosa, N., Aizpurua, X., and James, S.J. (2005). *International Journal of Refrigeration*, 28(6): 933-939.
- Jensen, K.N., Guldager, H.S., and Jørgensen, B.M. (2002). *Journal of Aquatic Food Product Technology*, 11(3/4): 201-214.

- Jensen, K.N. and Jørgensen, B.M. (2003). *Lebensmittel-Wissenschaft und -Technologie*, 36(8): 807-812.
- Jepsen, S.M., Pedersen, H.T., and Engelsen, S.B. (1999). *Journal of the Science of Food and Agriculture*, 79(13): 1793-1802.
- Kasapis, S., Rahman, M.S., Guizani, N., and Al-Aamri, M. (2000). *Journal of Agricultural and Food Chemistry*, 48(9): 3779-3784.
- Ke, P.J., Nash, D.M., and Ackman, R.G. (1976). *Canadian Institute of Food Science and Technology Journal*, 9(3): 135-138.
- Khayat, A. and Schwall, D. (1983). *Food Technology*, 37(7): 130-140.
- Kiran Jasra, S., Jasra, P.K., and Talesara, C.L. (2001). *Journal of the Science of Food and Agriculture*, 81(5): 519-524.
- Kjaersgard, I.V.H., Norrelykke, M.R., Baron, C.P., and Jessen, F. (2006a). *Journal of Agricultural and Food Chemistry*, 54(25): 9437-9446.
- Kjaersgard, I.V.H., Norrelykke, M.R., and Jessen, F. (2006b). *Proteomics*, 6(5): 1606-1618.
- Kristoffersen, S., Tobiassen, T., Esaiassen, M., Olsson, G.B., Godvik, L.A., Seppola, M.A., and Olsen, R.L. (2006). *Aquaculture Research*, 37(15): 1556-1564.
- Labuza, T.P., TANNENBA.SR, and Karel, M. (1970). *Food Technology*, 24(5): 543-&.
- Lanari, M.C., Schaefer, D.M., and Scheller, K.K. (1995). *Meat Science*, 41(3): 237-250.
- Leblanc, E.L. and Leblanc, R.J. (1988). *Journal of Food Processing and Preservation*, 12(2): 95-113.
- Leelapongwattana, K., Benjakul, S., Visessanguan, W., and Howell, N.K. (2008). *European Food Research and Technology*, 226(4): 833-841.
- Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., and Stadtman, E.R. (1990). *Methods in Enzymology*, 186: 464-478.
- Levine, R.L., Williams, J.A., Stadtman, E.R., and Shacter, E. (1994). *Oxygen Radicals in Biological Systems, Pt C*, 233: 346-357.
- Licciardello, J.J., Ravesi, E.M., Lundstrom, R.C., Wilhelm, K.A., Correia, F.F., and Allsup, M.G. (1982). *Journal of Food Quality*, 5(3): 215-234.

- Love, R.M. 1970. Ice formation in frozen muscle. In: Low temperature biology of food stuffs. Hawthorn, J. and Rolfe, E. J. (Ed.) Pergamon Press, Oxford. pp. 105-124.
- Love, R.M. and Haraldsson, S.B. (1961). *Journal of the Science of Food and Agriculture*, 12(6): 442.
- Mackie, I.M. (1993). *Food Reviews International*, 9(4): 575-610.
- Martino, M.N., Otero, L., Sanz, P.D., and Zaritzky, N.E. (1998). *Meat Science*, 50(3): 303-313.
- Miyashita, K. and Takagi, T. (1986). *Journal of the American Oil Chemists Society*, 63(10): 1380-1384.
- Morkore, T. and Lilleholt, R. (2007). *Journal of Texture Studies*, 38(4): 457-472.
- Nielsen, J. and Jessen, F. 2007. Quality of Frozen Fish. In: Handbook of Meat, Poultry and Seafood Quality. Nollet, L. M. L. (Ed.) Blackwell Publishing, Iowa. pp. 577-586.
- Nielsen, M.K. and Jørgensen, B.M. (2004). *Journal of Agricultural and Food Chemistry*, 52(12): 3814-3822.
- Nilsson, K. and Ekstrand, B. (1995). *Journal of Food Science*, 60(3): 627-630.
- Nott, K.P., Evans, S.D., and Hall, L.D. (1999). *Magnetic Resonance Imaging*, 17(3): 445-455.
- Orlien, V., Andersen, M.L., Jouhtimaki, S., Risbo, T., and Skibsted, L.H. (2004). *Journal of Agricultural and Food Chemistry*, 52(8): 2269-2276.
- Pham, Q.T. (1987). *Journal of Food Science*, 52(1): 210-212.
- Rahman, M.S. 1995. Food Properties Handbook. In: CRC Press, Boca Raton, FL. pp. 87-177.
- Rahman, M.S. 2001. Thermophysical properties of foods. In: Advances in food refrigeration. Sun, D.-W. (Ed.) Leatherhead Publishing, Surrey. pp. 70-109.
- Rahman, M.S. and Driscoll, R.H. (1994). *International Journal of Food Science and Technology*, 29(1): 51-61.
- Rahman, M.S., Kasapis, S., Guizani, N., and Al-Amri, O.S. (2003). *Journal of Food Engineering*, 57(4): 321-326.
- Riedel, L. (1956). *Kaeltechnik*, 8(12): 374-377.

- Riedel, L. (1957). *Kaeltechnik*, 9: 38-40.
- Rodriguez, A., Losada, V., Larrain, M.A., Quitral, V., Vinagre, J., and Aubourg, S. (2007). *Journal of the American Oil and Chemists Society*, 84: 727-734.
- Roos, Y.H. (1986). *Journal of Food Science*, 51(3): 684-686.
- Ross, K.D. (1978). *Journal of Food Science*, 43: 1812-1815.
- Ruan, R.R. and Chen, P.L. 1998. Aspects of water in food and biological systems. In: Water in foods and biological materials: A nuclear magnetic resonance approach. Ruan, R. R. and Chen, P. L. (Ed.) Technomic Publishing Company, Inc., Pennsylvania. pp. 51-74.
- Sablani, S.S., Kasapis, S., Rahman, M.S., Al-Jabri, A., and Al-Habsi, N. (2004). *Food Research International*, 37(10): 915-924.
- Sablani, S.S., Rahman, M.S., Al-Busaidi, S., Guizani, N., Al-Habsi, N., Al-Belushi, R., and Soussi, B. (2007). *Thermochimica Acta*, 462(1-2): 56-63.
- Saeed, S., Fawthrop, S.A., and Howell, N.K. (1999). *Journal of the Science of Food and Agriculture*, 79(13): 1809-1816.
- Saeed, S. and Howell, N.K. (1999). *Journal of the American Oil Chemists Society*, 76(3): 391-397.
- Schmidt, S.J. 2007. Water mobility in foods. In: Water activity in foods. Barbosa-Cánovas, G. V., Fontana, A. J., Jr., Schmidt, S. J. and Labuza, T. P. (Ed.) Blackwell Publishing and the Institute of Food Technologists, 47-108.
- Schnepf, M. 1989. Protein-water interactions. In: Water and food quality. Hardman, T. M. (Ed.) Elsevier Science Publishers Ltd., Essex. pp. 135-168.
- Schubring, R. (2004). *Deutsche Lebensmittel-Rundschau*, 100(7): 247-254.
- Schubring, R. (2005). *Deutsche Lebensmittel-Rundschau*, 101(11): 484-493.
- Scott, W.J. (1953). *Australian Journal of Biological Sciences*, 6(4): 549-564.
- Sei, T. and Gonda, T. (2006). *Journal of Crystal Growth*, 293(1): 110-112.
- Shenouda, S.Y.K. (1980). *Advances in Food Research*, 26: 311.

- Sigholt, T., Erikson, U., Rustad, T., Johansen, S., Nordtvedt, T.S., and Seland, A. (1997). *Journal of Food Science*, 62(4): 898-905.
- Sikorski, Z. 1994. Changes in proteins in frozen stored fish. In: Seafood proteins. Sikorski, Z., Pan, B. S. and Shahidi, F. (Ed.) Chapman & Hall, Inc., New York. pp. 99-112.
- Sikorski, Z.E. (1978). *International Journal of Refrigeration*, 1(3): 173-180.
- Sikorski, Z.E. and Kolakowska, A. 1994. Changes in Proteins in Frozen Stored Fish. In: Seafood Proteins. Sikorski, Z. E., Pan, B. S. and Shahidi, F. (Ed.) Chapman and Hall, New York. pp. 99-112.
- Sikorski, Z.E., Kostuch, S., and Olley, J. (1976). *Critical Reviews in Food Science and Nutrition*, 8(1): 97-129.
- Simatos, D., Faure, M., Bonjour, E., and Couach, M. (1975). *Cryobiology*, 12(3): 202-208.
- Simonides, W.S. and Vanhardeveld, C. (1990). *Analytical Biochemistry*, 191(2): 321-331.
- Slade, L. and Levine, H. (1991). *Critical Reviews in Food Science and Nutrition*, 30(2-3): 115-360.
- Slade, L. and Levine, H. (1995). *Advances in Food and Nutrition Research*, 103-269.
- Sørensen, N.K., Brataas, R., Nyvold, T.E. and Lauritzen, K. 1995. Influence of early processing (pre-rigor) on fish quality. In: Seafood from producer to consumer, integrated approach to quality. Luten, J. B., Børresen, T. and Oehlenschläger, J. (Ed.) Elsevier Science B.V., Amsterdam. pp. 253-263.
- Sotelo, C.G., Aubourg, S.P., Perez-Martin, R.I., and Gallardo, J.M. (1994). *Food Chemistry*, 50(3): 267-275.
- Steen, C. and Lambelet, P. (1997). *Journal of the Science of Food and Agriculture*, 75(2): 268-272.
- Tokunaga, T. (1974). *Bulletin of the Japanese Society of Scientific Fisheries*, 40(2): 167-174.
- Trout, G.R. (1988). *Meat Science*, 23(4): 235-252.
- Van der Berg, C. 1991. Food-water relations: progress and integration, comments and thoughts. In: Water relationships in foods - advances in the 1980s and trends in the 1990s. Levine, H. and Slade, L. (Ed.) Plenum Press, New York. pp. 21-28.
- Vyncke, W. (1970). *Fette Seifen Anstrichmittel*, 72(12): 1084-1087.



- Watabe, S. and Hashimoto, K. (1986). *Food Reviews International*, 2(3): 353-393.
- Wolfe, J. and Bryant, G. (2001). *International Journal of Refrigeration*, 24(5): 438-450.
- Wolfe, J., Bryant, G., and Koster, K.L. (2002). *Cryoletters*, 23(3): 157-166.
- Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y., and Yamamoto, K. (1999). *Journal of Biochemistry*, 125: 1137-1143.
- Yoshida, H., Kondo, I., and Kajimoto, G. (1992). *Journal of the American Oil Chemists Society*, 69(11): 1136-1140.



## Paper I

---

Effect of temperature on quality-related changes in cod  
(*Gadus morhua*) during short- and long-term frozen storage.

Burgaard and Jørgensen (2010).

Submitted to *Journal of Aquatic Food Product Technology*.

---

**Effect of temperature on quality-related changes in cod (*Gadus morhua*) during short- and long-term frozen storage.**

Keywords: Freezing, Fish, Quality indicators, ULF

Running title: Effect of temperature on frozen-stored cod quality

***ABSTRACT***

Cod (*Gadus morhua*) was stored at eight temperatures (-10 to -80 °C) from one to 18 months after which some quality indicators were measured: drip loss, water holding capacity, low field NMR spin-spin relaxation, colour, amount of thiobarbituric acid reactive substances and sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase and lysosomal Cathepsin D activities. Results from samples stored up to 12 months showed no significant difference between -30 °C or lower temperatures. The NMR measurements, however, indicated some changes in the water distribution of samples stored at -30 °C for 12 or more months compared to storage at -40 °C or lower.

## ***INTRODUCTION***

Freezing is an important preservation method, and an increasing number of trawlers are equipped with freezers, thus the fish can be frozen and stored immediately after catch. This gives an opportunity to prolong the season and provide raw material to the filleting industry in times of small or no catches. If the frozen fish is stored at low, non-fluctuating temperatures and thawed in the best way according to its rigor-state, the quality can be as good as or better than fresh fish stored for a few days at 0 °C (Cappeln et al., 1999). For optimal handled cod, the quality remains as high as for fresh cod for one month at -30 °C. The fish is still suitable for consumption after one year, though the characteristic frozen storage flavour starts to develop after approximately three months (Nielsen and Jessen, 2007).

Freezing and frozen storage of the fish can lead to structural and physicochemical changes that alter the properties of the fish muscle in a way that can cause quality deteriorations to different degrees. The most common deteriorating processes, which take place in fish during frozen storage, are: physical alterations in the texture caused by large ice crystals (protein denaturation), freezer burn, lipid oxidation, protein oxidation and discolorations. These processes take place to different degrees dependent on fish species, freezing and thawing rate, storage temperature, storage time and protection against light and oxygen (Sikorski et al., 1976; Sikorski, 1978; Cappeln et al., 1999; Careche et al., 1999; Herrera et al., 2001; Leelapongwattana et al., 2005).

At temperatures just below the freezing point of fish muscle, salts and enzymes become more concentrated, due to crystallisation of water. This can cause myofibrillar proteins to denature and aggregate with hard, dry and fibrous fish material as a result. According to Buttkus (1970), aggregation of myosin in trout is higher at -10 °C compared to 0, -20 and -30 °C. The rates of lipid and protein oxidation are also increased compared to unfrozen muscle despite the lower temperature (Buttkus, 1970; Sikorski and Kolakowska, 1994).

The rate of freezing also has great impact on the rate of several quality-deteriorating processes. Slow freezing and fluctuating temperatures lead to formation of fewer and larger ice crystals on the expense of the many small that are typically formed during fast freezing. The formation of large extra-cellular ice crystals leads to cells dehydrated to an extent that intra-cellular solutions with very high concentrations of enzymes and salts appear. This increases reaction rates further, but as the temperature falls, viscosity rises and slows the

reaction rates down again (Love, 1968; Wolfe and Bryant, 2001). Moreover, the formation of large ice crystals results in physical damages to proteins, cell walls etc., since the extra cellular ice occupy far more space than the original extra cellular spaces and thus compress the dehydrated cells (Love, 1968).

Some of the damage to cells and proteins caused by freezing can be diminished using higher freezing rates and by preventing fluctuating storage temperatures. Thereby many small ice crystals, both intra- and extra-cellular, are formed and the cells are not dehydrated because ice crystals are formed before water has time to diffuse out of the cell (Love, 1968). Fast freezing rates may also increase water holding capacity thus increasing the quality of the product. In cod, sensory quality and liquid holding capacity has been shown to correlate very well (Nielsen and Jessen, 2007).

In gadoid species, such as cod and hake, another problem is associated with freezing and frozen storage. The formation of formaldehyde and dimethylamine (DMA), from the enzymatic breakdown of trimethylamine oxide (TMAO), can lead to extensive textural changes, probably due to cross-linking of proteins. The increase in concentration of formaldehyde and DMA during frozen storage is highest around -10 °C and negligible around -26 to -30 °C and lower except for red and white hake, which are probably the most unstable of the gadoid fish (Castell et al., 1970; Babbitt et al., 1972; Tokunaga, 1974; Dingle et al., 1977; Leblanc and Leblanc, 1988; Chapman et al., 1993; Careche et al., 1998; Careche et al., 1999). Some authors (Badii and Howell, 2002a; Badii and Howell, 2002b) claim, however, that formaldehyde is not a major factor responsible for changes in texture and proteins in frozen cod and haddock fillets.

Most previous studies have focused on the effect of different storage temperatures in the interval between 0 and -30 °C on the quality of fish. As mentioned, this temperature interval is very important when it comes to storage of gadoid fish species and it is well known that storage at approximately -30 °C compared to storage at -20 °C or higher results in products of higher quality, also in non-formaldehyde producing species. The effect of temperatures lower than -30 °C is primarily studied in relation to tuna quality, especially colour (Watabe and Hashimoto, 1986; Chow et al., 1988) though, or with the use of one low temperature (-40 or -80 °C) for reference purpose only.

In the present study, we conducted a comprehensive investigation on the relation between loss of quality, storage time and storage temperature in the interval between -10 and -80 °C and storage times up to 18 months. Storage at -10 °C was included in order to have a 'negative' reference, because storage of fish at this temperature is known to cause extensive quality loss. The quality-related properties measured were: drip loss, water holding capacity, colour, lipid oxidation expressed as content of thiobarbituric acid-reactive substances (TBARS) and low-field nuclear magnetic resonance (NMR) spectroscopy, which gives information about the distribution of water in the muscle. The activities of the enzymes Sarcoplasmic Reticulum  $\text{Ca}^{2+}$ -ATPase (EC 3.6.1.38) and Cathepsin D (EC 3.4.23.5), were used as indicators for the integrity of the sarcoplasmic reticulum membrane and the lysosomes, respectively.

## ***MATERIALS AND METHODS***

### ***Experimental design***

North Sea cod (*Gadus morhua*) were caught in trawl in February 2007 and transported on ice to DTU Aqua not later than two days after catch. 22 cod (5 kg each) were filleted by hand on day three after slaughtering. The loin parts of the fillets were cut in four pieces each, vacuum packed, frozen in a -40 °C blast freezer and randomly distributed to eight different freezers at temperatures between -10 and -80 °C (10 °C intervals). The fish were freeze stored from one to 18 months (1, 2, 3, 4, 5, 6, 8, 10, 12, 15 and 18). In order to investigate the effect of different storage temperatures, the experimental design was paired regarding temperature, so that pieces stored for one month at the eight different temperatures originated from the same fish. For each storage period, two different fish were examined.



### *Analyses*

After each storage period, the cod were thawed in a 2 °C cold store overnight (samples for drip loss, water content, water holding capacity and NMR) or in 8 °C water (samples for the other analyses) and the following analyses were performed: low-field NMR- measurements, thiobarbituric acid reactive substances (TBARS), drip loss, water holding capacity, water content, colour measurements and activity of Cathepsin D and  $\text{Ca}^{2+}$ -ATPase. Lipid content and fatty acid composition were measured on samples stored at -80 °C. For some of the analyses, cod fillet was minced for 5 s in a meat mincer (Foss Tecator Knifetec 1095 Sample Mill, Hillerød, Denmark). All analyses were made in duplicate unless otherwise indicated.

#### *Drip loss, water content and water holding capacity*

Fillets were weighed before and after thawing, and the drip loss was measured as the loss in mass divided by the initial mass of the fillet. Before weighing the thawed fillets the excess drip from the surface was wiped off using a paper towel.

The water content was measured as the loss in mass after drying 2 g sample overnight at 105 °C divided by the original sample mass.

The water holding capacity was defined as the capacity of the minced muscle to hold its own cellular fluid when centrifuged (Eide et al., 1982). Portions of carefully mixed mince (2 g) were weighted into cylindrical tubes whose bottom was a filter allowing liquid but not solid material to pass. The tubes were then placed in 50 ml centrifuge tubes equipped with approximately 40 g glass marbles to provide space for the filtrate. Centrifugation was performed at 10 °C for 5 min at 1500 g in a Sigma 4K15 centrifuge equipped with a 12166 rotor. The water holding capacity was expressed as the mass of water remaining in the muscle sample after centrifugation divided by the mass of water in the original sample.

#### *Nuclear magnetic resonance*

The NMR-measurements were performed on portions of 2-3 g mince that were weighted into small cylindrical glass tubes fitting in the 18 mm NMR sample tubes. The samples were equilibrated for 30 min in an 8 °C water bath before measurements. Low-field relaxation measurements were performed on a Maran Benchtop Pulsed NMR analyser (Resonance Instruments, Witney, UK) operating at 23.2 MHz and equipped with an 18 mm variable temperature probe head. Transverse relaxation was measured at 8 °C as described by Jensen

et al.(Jensen et al., 2002) using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. The 180°-pulse spacing ( $2\tau$ ) was 400  $\mu$ s and the number of data points acquired was 1024, spaced by  $4\tau$  (even echoes sampled).

### ***Enzyme activity***

Homogenates for determination of  $\text{Ca}^{2+}$ -ATPase and Cathepsin D activity were prepared from 3.0 g muscle cut into small pieces in 24 ml cold 10 mM Tris-HCl buffer (pH 7.0) containing 0.25 M sucrose. A Potter-Elvehjem apparatus was used to homogenise the suspension by 10 up and down strokes with a Teflon pestle at 750 rpm. Afterwards the homogenate was centrifuged at 1000 g (4 °C) for 15 min and the supernatant was kept on ice or frozen at -80 °C until use.

The activity of  $\text{Ca}^{2+}$ -ATPase was measured using the method described by Godiksen and Jessen (2001), based on the work of Simonides and Hardeveld (1990). The activity of  $\text{Ca}^{2+}$ -ATPase was defined as the difference between liberated inorganic phosphate from ATP in the presence (total activity) and absence (basal activity) of free  $\text{Ca}^{2+}$ .

Cathepsin D activity in the homogenates was assayed using a slightly modified method of Yasuda et al. (Yasuda et al., 1999), based on the fluorescence of the (7-methoxycoumarin-4-yl)acetyl (MOCAC) moiety which is splitted from the synthetic substrate MOCAC-Gly-Lys-Pro-Ile-Leu-Phe~Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH<sub>2</sub> catalysed by Cathepsin D. The substrate was prepared from 1.0 mg MOCAC-Gly-Lys-Pro-Ile-Leu-Phe~Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH<sub>2</sub> dissolved in 200  $\mu$ l acetate buffer (50 mM sodium acetate, pH 4.0) and 369  $\mu$ l dimethylsulfoxid (DMSO) and thereafter diluted 4 times in the same acetate buffer. The final concentration of DMSO in the assay was 1.28% and the substrate concentration 200  $\mu$ M. 80  $\mu$ l acetate buffer with 1 mM EDTA was added to the wells of a black assay plate (Corning Incorporated, NY) and heated for 15 min in a fluorescence spectrophotometer (Shimadzu UV-160A) at 35 °C. 10  $\mu$ l of sample solution (supernatant) and 10  $\mu$ l of substrate were added and the fluorescence of the cut substrate measured at an emission wavelength of 393 nm with excitation at 328 nm for 10 min. The slope of the curve fluorescence *versus* time was used as a measure of the activity of the enzyme.

### ***Thiobarbituric acid-reactive substances (TBARS)***

TBARS was determined according to the method of Vyncke (1970) with the modification that the reaction was done at 90 °C instead of in boiling water.

### ***Colour***

Colour parameters (CIE 1976,  $L^*$ ,  $a^*$ ,  $b^*$  colour space) were measured by a Minolta Chroma Meter CR-200 (Konica Minolta Sensing Inc., Japan). The values reported are average of triplicate measurements at two or three different measuring points on the flesh side of the fillet depending on its size.

### ***Lipid content and fatty acid composition***

The lipid content of the cod samples was determined by the method of Bligh and Dyer (Bligh and Dyer, 1959) slightly modified by reducing solvent volumes. Fatty acid methyl esters were prepared using an AOCS official method (Ce 2-66, 1998) and analysed by gaschromatography, also using an official AOCS method (Ce 1 b-89, 1998). A HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an Omegawax 320 column (30m  $\times$  3.2mm, 0.25  $\mu$ m; Supelco, Bellefonte, PA, USA) and a flame ionization detector (Hewlett-Packard, Palo Alto, CA, USA) was used for the analyses. The fatty acids were identified by comparison with chromatograms of standards.

### ***Data analysis***

Univariate one- and two-way analysis of variance (ANOVA) were used to look for possible differences in quality parameters due to temperature or time. According to the experimental design, repeated measures ANOVA was used between groups at different temperatures and a Bonferroni post test was used to compare all pairs of columns. Test for linear trend was made using regression on the temperature variable. The multivariate analysis methods principal component analysis (PCA) and partial least squares (PLS) regression were applied to the NMR relaxation curves with data from samples as rows in the  $X$ -matrix and (for the PLS regression) water holding capacity as  $y$ . Each column in the data matrix was mean-centred. The multivariate models were cross-validated using 2 segments, each with 88 members (samples from 11 different fish, stored for 11 different periods in each segment).

## ***RESULTS AND DISCUSSION***

The average lipid content of the 22 cod was  $0.7 \pm 0.1$  % and the water content was  $79.7 \pm 1.3$  %. There were no significant differences in the polyunsaturated fatty acid composition of the 22 cod (results not shown).

***Drip loss and water holding capacity***

The drip loss of cod was highest for samples stored at high temperatures and decreased with decreasing temperature ( $p < 0.0001$  in the test for linear trend, Table 1), and generally samples stored at -10 and -20 °C had a higher drip loss than samples stored at lower temperatures. The water holding capacity (WHC) during storage at -10 to -80 °C for one to 18 months is shown in Table 2. The WHC of samples stored at -10 and -20 °C decreased during frozen storage and, as expected, samples stored at -10 °C had lower WHC compared to all other temperatures at all times except for one of the samples stored in a broken bag at -80 °C for four months. After five months and during the rest of the experiment (except at 8 months) samples stored at -20 °C had significantly lower WHC than samples stored at -30 to -80 °C. No difference was found between WHC in samples stored at temperatures from -30 to -80 °C during the storage times used. Mørkøre and Lilleholt (2007) also reported a higher drip loss in cod frozen and stored at -10 and -20 °C compared to -40 to -70 °C. Their cod were only stored for 10-11 days, so the temperature during freezing probably had a more pronounced effect than the storage temperature. The increased drip loss and decreased WHC for samples stored -10 and -20 °C were possibly a result of protein changes leading to altered functional properties. This may, at least partly, be caused by ice crystal growth and protein and lipid oxidation. Another factor could be formation of dimethylamine and formaldehyde from trimethylamine-N-oxide, (Nielsen and Jørgensen, 2004) as formaldehyde may form cross-links between proteins resulting in decreased protein solubility, aggregation and thereby loss of functional properties (Castell et al., 1970; Babbitt et al., 1972; Sikorski, 1978; Gill et al., 1979).

***NMR***

The NMR relaxation curves contain information on the water content and distribution in the muscle. By normalising the signal to common total amplitude, contributions from a trivial difference in water content is removed. Thus, changes in curve shapes reflect changes in water distribution only. A principal component analysis (PCA) showed that samples stored at -10 °C as expected were most different from other samples. When removing the -10 °C samples from the data set and redoing the PCA, also samples stored at -20 °C showed up as different from the rest (Fig. 1). However, they did not differ from samples stored at lower temperatures until after approximately 4-5 months of frozen storage. Steen and Lambelet

(1997) showed that cod mince stored at -10 °C for 4 months had a larger proportion of slowly relaxing water compared to samples stored at -20 and -70 °C; moreover the proportion of slowly relaxing water increased with increasing storage temperature. Jensen et al. (2003) also showed that for cod samples stored for at least three months, storage at -20 °C resulted in a higher proportion of slowly relaxing water compared to -30 °C. In the present study, samples stored at -30 °C did not differ from samples stored at -40 to -80 °C for the first 10 months but after 12 months of frozen storage they differed from samples stored at lower temperatures. The change in water distribution as measured by the NMR technique for samples stored at -30 °C after 12 months is probably caused by textural changes affecting water distribution or binding. Steen and Lambelet (1997) showed very high correlations between NMR  $T_2$ -values and several analytical methods (instrumental and sensory) for determining texture of cod mince. Also, lean fish species are claimed to have a storage life (consumer acceptability) of at least 12 months at -30 °C (Almandos et al., 1984; Chapman et al., 1993; Nielsen and Jessen, 2007), which is in accordance with the results of the NMR measurements in the present study. It was not possible to see any differences caused by storage temperature in samples stored at -40 to -80 °C during 18 months of frozen storage.

Using the NMR-relaxation curves as x-variables in a PLS regression, the WHC of the samples were predicted. Predicted and measured WHC correlated well ( $r = 0.86$ ,  $n=176$ ) and the results of the two methods were very similar. This relationship was also shown by Andersen and Jørgensen (2004) and Jepsen et al. (1999).

#### ***Ca<sup>2+</sup>-ATPase activity***

Cod stored at -10 and -20 °C generally had significantly lower Ca<sup>2+</sup>-ATPase activity than samples stored at -40 °C or lower (1-way ANOVA, Fig. 2). Hsu et al. (1993) measured the Ca<sup>2+</sup>-ATPase activity of actomyosin in Pacific Whiting and reported a more rapid decrease of enzyme activity for samples stored at -8 °C compared to samples stored at -20, -34 and -50 °C. Godiksen et al. (2003) were able to differentiate fish stored at -30 (highest activity) and -20 °C after five months of frozen storage. This is in fair agreement with our results as some differences can be expected due to freezing and thawing method and variation between individuals. For samples stored at temperatures between -30 and -80 °C there were a tendency to increased enzyme activity after approximately two months of frozen storage. Inoue et al. (1992) measured Ca<sup>2+</sup>-ATPase activity of frozen carp myosin and also reported an increased

activity for samples stored at -15 and -20 °C during the first 12 hours of frozen storage. The activity of samples stored at -5 to -13 °C decreased. Godiksen et al. (2003) also reported on activation of  $\text{Ca}^{2+}$ -ATPase activity as a result of freezing and suggested that the freeze-induced activation of the enzyme was due to changed interactions between  $\text{Ca}^{2+}$ -ATPase and the surrounding membrane lipids.

### ***Cathepsin D activity***

During storage from one to 18 months, cod samples stored at -10 and -20 °C generally had significantly lower enzyme activities than samples stored at lower temperatures (Table 3). Large variations in activity over time were observed which might be due to variation between individual fish. Another possibility is that an increased activity caused by release of Cathepsin D to the tissue from lysosomes as a result of destroyed cell membranes competed with a decreased activity over time due to protein denaturation and/or other protease activities.

### ***TBARS***

Samples stored at -10 °C generally had higher TBARS content than had samples stored at lower temperatures (1 way ANOVA, Table 4), but there were no differences between samples stored at -20 °C and lower. This is in fair agreement with Dulavik et al. (1998), who reported on higher TBA-values in dark muscle of saithe stored for nine months at -10 and -20 °C compared to samples stored at -30 and -80 °C, though only a minor increase were observed in light muscle stored at -10 °C compared to the lower temperatures. Also, Aubourg and Medina (1999) observed a small but significant difference in TBARS content between frozen stored cod and haddock stored for up to 12 months at -10 and -30 °C.

### ***Colour***

The results of the colour measurements are shown in Figure 3. Values for  $L^*$ ,  $a^*$  and  $b^*$  were analysed using 1-way ANOVA. Cod samples stored at -10 and -20 °C were significantly more light (higher  $L^*$ ) and yellowish (negative  $b^*$ ) than samples stored at lower temperatures. For samples stored at -10 °C there was a tendency towards higher  $b^*$ -values with increasing storage times. Moreover, samples stored at -10 °C had lower  $a^*$ -values than samples stored at lower temperatures, indicating a more greenish colour. Similar results for changes in  $L^*$ - and  $b^*$ -values in frozen stored cod were obtained in a study by Schubring, who in one study (Schubring, 2005) observed increasing  $L^*$ - and  $b^*$ -values for samples stored at -20 and especially -14 °C compared to samples stored at -28 °C and in another (Schubring, 2004)

increasing  $b^*$  - and  $L^*$  -values and decreasing a-values for -10 °C compared to -20 and -30 °C. The colour changes in cod include loss of surface glossiness, muscle opacity or chalky appearance and are thought to be due to irreversible changes in the muscle proteins (Shenouda, 1980).

### ***CONCLUSION***

A storage temperature of -30 °C seems to be sufficiently low for preservation of post rigor frozen cod up to 12 months. The results of the WHC and NMR measurements indicate that long time storage (> 12 months) at -40 °C or lower compared to -30 °C or higher results in less pronounced changes in water holding capacity and water distribution and thereby probably also less protein denaturation. For this type of fish products, no advantage in keeping the quality was found by using storage temperatures below -30 °C.

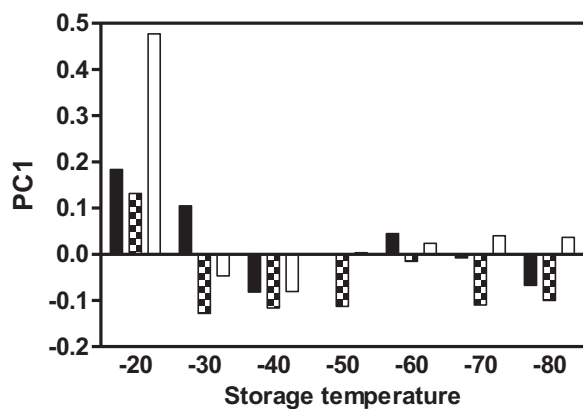
**REFERENCES**

- Almandos, M.E., Giannini, D.H., Ciarlo, A.S., and Boeri, R.L. 1984. Lebensmittel-Wissenschaft und -Technologie. 17(2): 111-113.
- Andersen, C.M. and Jørgensen, B.M. 2004. J. Aquat. Food Prod. Technol. 13(1): 13-23.
- AOCS. 1998. Official Method Ce 1 b-89. Fatty Acid Composition by GLC. Marine Oils. AOCS, Champaign, IL.
- AOCS. 1998. Official Method Ce 2-66. Preparation of methyl esters of fatty acids. AOCS, Champaign, IL.
- Aubourg, S.P. and Medina, I. 1999. J. Sci. Food Agric. 79(13): 1943-1948.
- Babbitt, J.K., Crawford, D.L., and Law, D.K. 1972. J. Agric.Food Chem. 20(5): 1052-1054.
- Badii, F. and Howell, N.K. 2002a. J. Sci. Food Agric. 82(1): 87-97.
- Badii, F. and Howell, N.K. 2002b. Food Hydrocolloids. 16(4): 313-319.
- Bligh, E.G. and Dyer, W.J. 1959. Can. J. Biochem. Physiol. 37: 911-917.
- Buttkus, H. 1970. J. Food Sci. 35: 558-562.
- Cappeln, G., Nielsen, J., and Jessen, F. 1999. J. Sci. Food Agric. 79(8): 1099-1104.
- Careche, M., del Mazo, M.L., Torrejon, P., and Tejada, M. 1998. J. Agric.Food Chem. 46(4): 1539-1546.
- Careche, M., Herrero, A.M., Rodriguez-Casado, A., del Mazo, M.L., and Carmona, P. 1999. J. Agric.Food Chem. 47(3): 952-959.
- Castell, C.H., Neal, W., and Smith, B. 1970. J. Fisheries Res. Board Can. 15: 701-716.
- Chapman, K.W., Ifat, S., Keum Taek, H., and Regenstein, J.M. 1993. J. Food Sci. 58(6): 1208-1211.
- Chow, C.J., Ochiai, Y., Watabe, S., and Hashimoto, K. 1988. Bull. Jap. Soc. Sci. Fisheries 54(4): 639-648.
- Dingle, J.R., Keith, R.A., and Lall, B. 1977. Can. Inst. Food Sci. Technol. J. 10(3): 143-146.



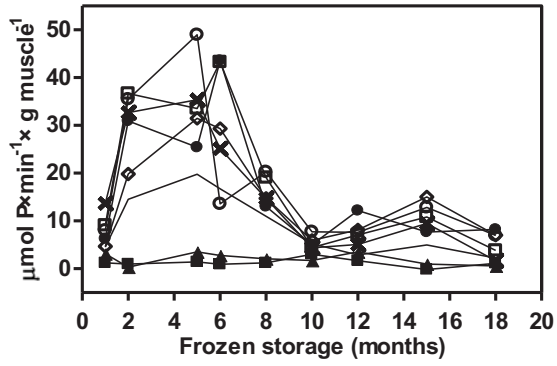
- Dulavik, B., Sorensen, N.K., Barstad, H., Horvli, O., and Olsen, R.L. 1998. *J. Food Lipids* 5(3): 233-245.
- Eide, O., Børresen, T., and Strøm, T. 1982. *J. Food Sci.* 47: 347-349, 354.
- Gill, T.A., Keith, R.A., and Lall, B.S. 1979. *J. Food Sci.* 44(3): 661-667.
- Godiksen, H., Hyldig, G., and Jessen, F. 2003. *J. Food Sci.* 68(8): 2579-2585.
- Godiksen, H. and Jessen, F. 2001. *J. Food Biochem.* 25(4): 343-358.
- Herrera, J.J., Pastoriza, L., and Sampedro, G. 2001. *J. Sci.Food Agric.* 81(4): 377-384.
- Hsu, C.K., Kolbe, E., Morrissey, M.T., and Chung, Y.C. 1993. *J. Food Sci.* 58(5): 1055-1056.
- Inoue, N., Takatori, K., Motoshige, T., and Shinano, H. 1992. *Bull. Jap. Soc. Sci. Fisheries* 58(12): 2357-2360.
- Jensen, K.N., Guldager, H.S., and Jørgensen, B.M. 2002. *J. Aquat. Food Prod. Technol.* 11(3/4): 201-214.
- Jensen, K.N. and Jørgensen, B.M. 2003. *Lebensm.-Wissens. Technol.* 36(8): 807-812.
- Jepsen, S.M., Pedersen, H.T., and Engelsen, S.B. 1999. *J. Sci. Food Agric.* 79(13): 1793-1802.
- Leblanc, E.L. and Leblanc, R.J. 1988. *J. Food Process. Preserv.* 12(2): 95-113.
- Leelapongwattana, K., Benjakul, S., Visessanguan, W., and Howell, N.K. 2005. *Food Chem.* 90(1-2): 141-150.
- Love, R.M. 1968. Ice Formation in Frozen Muscle. In: *Low Temperature Biology of Foodstuffs*. Hawthorn, J. and Rolfe, E. J. (Ed.) Pergamon Press, Oxford, 105-124.
- Morkore, T. and Lilleholt, R. 2007. *J. Text. Stud.* 38(4): 457-472.
- Nielsen, J. and Jessen, F. 2007. Quality of Frozen Fish. In: *Handbook of Meat, Poultry and Seafood Quality*. Nollet, L. M. L. (Ed.) Blackwell Publishing, Iowa. pp. 577-586.
- Nielsen, M.K. and Jørgensen, B.M. 2004. *J. Agric.Food Chem.* 52(12): 3814-3822.
- Schubring, R. 2004. *Deutsche Lebensmittel-Rundschau.* 100(7): 247-254.

- Schubring, R. 2005. Deutsche Lebensmittel-Rundschau. 101(11): 484-493.
- Shenouda, S.Y.K. 1980. Adv. Food Res. 26: 311.
- Sikorski, Z.E. 1978. Int. J. Refrig. 1(3): 173-180.
- Sikorski, Z.E. and Kolakowska, A. 1994. Changes in Proteins in Frozen Stored Fish. In: Seafood Proteins. Sikorski, Z. E., Pan, B. S. and Shahidi, F. (Ed.) Chapman and Hall, New York. pp. 99-112.
- Sikorski, Z.E., Kostuch, S., and Olley, J. 1976. CRC Critical Reviews in Food Science and Nutrition. 8(1): 97-129.
- Simonides, W.S. and Vanhardeveld, C. 1990. Anal. Biochem. 191(2): 321-331.
- Steen, C. and Lambelet, P. 1997. J. Sci.Food Agric. 75(2): 268-272.
- Tokunaga, T. 1974. Bull. Jap. Soc. Sci. Fisheries 40(2): 167-174.
- Vyncke, W. 1970. Fette Seifen Anstrichmittel. 72(12): 1084-1087.
- Watabe, S. and Hashimoto, K. 1986. Food Rev. Int. 2(3): 353-393.
- Wolfe, J. and Bryant, G. 2001. Int. J. Refrig. 24(5): 438-450.
- Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y., and Yamamoto, K. 1999. J. Biochem. 125: 1137-1143.



**Figure 1.** PC1 scores from a PCA on NMR relaxation measurements.

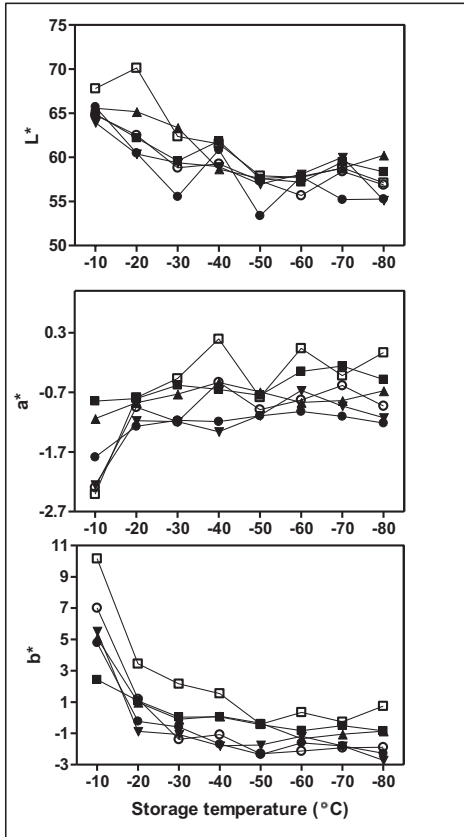
Samples from all storage times and temperatures used were included in the analysis. PC1 accounted for 89% of the total variation between samples. The different symbols represent different storage times: ■, 1 month; ▣, 6 months; □, 18 months.



**Figure 2.** Effect of time and temperature on  $\text{Ca}^{2+}$ -ATPase activity in frozen-stored cod.

Symbols used: ■, -10; ▲, -20; ▼, -30; ●, -40; ×, -50; ◇, -60; ○, -70; □, -80 °C.

2-way ANOVA: temperature,  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ ; all  $p$ -values are for analyses without -10 °C.

**Figure 3****Figure 3.** Colour of frozen stored cod samples as a function of frozen storage temperature.

Symbols used: ■, 5; ▲, 8; ▼, 10; ●, 12; ○, 15; □, 18 months.

1-way ANOVA without -10 °C:  $L^*$ ,  $p < 0.0001$ ;  $a^*$ ,  $p > 0.05$ ;  $b^*$ ,  $p = 0.001$ .

Table 1. Drip loss of cod fillet.

Temperature (°C)		Frozen storage (months)											
		1	2	3	4	5	6	8	10	12	15	18	
-10 <sup>a</sup>	1	15.26	12.17	8.11	12.57	14.03	8.11	15.23	15.57	14.59	12.38	17.75	
	2	11.74	14.99	12.32	14.27	11.58	12.32	16.70	14.43	13.07	11.52	15.77	
-20	1	14.12	15.02	12.58	12.31	15.31	12.58	11.55	13.21	12.09	14.41	19.01	
	2	13.24	14.49	12.00	10.69	13.37	12.00	12.98	20.32	18.77	15.84	15.08	
-30	1	9.74	13.34	9.83	10.77	10.74	9.83	7.42	11.20	11.97	14.26	11.11	
	2	11.88	13.87	12.93	11.70	13.93	12.93	12.13	10.65	14.28	15.40	13.68	
-40	1	10.71	12.62	6.45	7.60	9.04	6.45	8.42	12.06	10.76	16.65	13.02	
	2	8.45	9.37	9.68	8.11	12.55	9.68	14.69	12.04	13.79	8.80	11.58	
-50	1	14.43	7.78	6.92	9.79	12.63	6.92	8.27	11.89	11.34	14.12	13.90	
	2	7.18	12.49	11.43	12.01	13.36	11.43	11.86	15.89	15.27	12.82	16.13	
-60	1	7.72	13.36	10.25	7.28	10.47	10.25	4.54	8.03	8.32	11.64	12.54	
	2	10.82	12.81	7.42	12.56	8.54	7.42	10.01	5.94	15.52	10.77	14.31	
-70	1	7.10	11.53	8.73	8.26	5.58	8.73	9.44	9.78	11.00	16.30	11.02	
	2	10.24	10.30	8.28	7.44	5.43	8.28	12.41	14.69	13.09	9.23	7.56	
-80	1	6.90	9.34	5.30	13.31	10.29	5.30	7.68	6.95	10.32	6.40	12.08	
	2	9.20	8.29	6.99	7.12	11.41	6.99	9.18	8.16	2.24	9.83	12.17	
1-way ANOVA		$p > 0.05$		$p > 0.05$	$p > 0.05$	$p = 0.017$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	

a) All  $p$ -values are for analyses without -10 °C.

1-way ANOVA (all months);  $p < 0.0001$ .

Table 2. Water holding capacity of cod fillets.

Temperature (°C)	Frozen storage (months)																							
	1		2		3		4		5		6		8		10		12		15		18			
	Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
-10 <sup>b</sup>	1	53.65	10.94	47.30	11.70	33.54	8.76	26.53	3.51	26.69	5.88	35.53	2.61	50.60	12.71	30.59	6.14	20.19	7.15	-	60.11	0.29	7.82	0.33
	2	44.26	1.46	52.84	0.46	41.90	1.18	74.30	1.36	9.57	7.46	38.63	5.08	36.78	6.00	0.87	11.67	6.40	2.53	-0.03	4.60	12.40	5.04	
-20	1	80.39	4.37	79.89	2.93	67.71	7.98	70.32	2.14	68.52	1.53	67.59	3.29	78.99	3.21	51.96	0.46	63.81	3.67	49.80	3.07	41.01	7.83	
	2	75.22	0.81	82.39	3.79	73.05	1.06	81.66	2.25	61.97	7.19	69.13	15.44	75.57	5.59	66.43	1.13	52.92	2.78	44.00	0.71	34.69	7.10	
-30	1	78.48	0.12	80.12	8.64	73.53	0.01	82.03	1.34	84.63	0.06	86.05	2.29	90.10	1.19	83.59	0.71	79.59	3.59	75.52	0.99	71.01	2.56	
	2	74.34	2.15	82.16	2.75	90.24	2.19	87.45	1.13	80.89	1.98	88.26	4.16	83.85	1.58	84.24	1.44	83.05	2.60	83.63	3.20	78.65	6.20	
-40	1	86.31	3.59	85.74	1.80	82.21	6.54	87.77	7.62	91.27	3.23	89.35	0.72	93.99	0.03	92.88	1.89	90.46	1.62	81.46	1.24	81.71	0.82	
	2	87.15	1.19	90.97	3.46	88.16	4.19	91.35	3.03	74.98	2.15	86.62	1.81	89.96	3.29	87.13	2.35	90.67	2.09	89.52	3.57	87.62	0.29	
-50	1	82.29	2.50	85.81	3.24	75.27	3.34	82.79	3.48	88.69	0.27	88.16	1.18	86.75	2.16	86.94	2.10	91.07	1.06	84.62	2.51	80.39	3.99	
	2	87.82	2.55	83.53	3.59	79.80	2.49	81.65	1.08	83.36	1.90	92.54	2.90	90.96	2.04	84.79	2.33	86.55	1.45	88.52	0.94	82.54	1.75	
-60	1	89.86	1.16	84.83	3.47	77.89	1.36	83.64	4.43	86.83	1.38	87.75	2.64	89.50	1.04	93.42	0.09	94.17	1.64	76.97	1.09	74.24	1.59	
	2	82.48	3.28	87.88	4.17	87.61	3.17	82.64	2.95	80.92	2.83	85.14	0.59	88.45	2.15	89.58	1.33	86.40	2.23	89.53	1.46	83.52	1.22	
-70	1	83.56	1.94	82.31	1.48	73.04	0.08	87.65	1.24	85.87	0.96	92.62	1.86	91.50	0.19	94.96	1.19	93.22	0.87	77.01	1.19	85.87	2.59	
	2	84.17	6.88	86.57	4.30	85.41	2.25	88.09	3.01	79.34	1.94	89.34	0.26	91.58	2.17	88.64	3.41	90.53	1.87	93.61	0.66	78.96	2.49	
-80	1	87.00	4.06	88.79	0.16	81.96	1.06	34.51 <sup>*</sup>	2.28	91.57	0.18	91.01	0.84	91.09	3.83	82.37	4.97	93.53	1.18	82.80	2.75	85.71	3.42	
	2	83.58	0.34	88.37	0.65	88.03	1.47	83.27	0.62	80.86	3.25	90.78	0.75	90.66	0.51	82.73	0.08	90.64	1.74	88.25	6.57	80.39	2.72	
1-way ANOVA		$p > 0.05$		$p = 0.0287$		$p = 0.0309$		$p > 0.05$		$p = 0.0044$		$p = 0.0003$		$p = 0.0087$		$p = 0.0061$		$p = 0.0005$		$p = 0.0018$		$p = 0.0007$		

b) All  $p$ -values are for analyses without -10 °C.

\*) Stored in a broken bag.

2-way ANOVA: temperature,  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ .

Table 3. Cathepsin D activity in cod fillets.

Temperature (°C)		Frozen storage (months)																	
		1		2		3		4		5		6		8		10		12	
		Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
-10 <sup>b</sup>	1	1.58	0.14	1.37	0.20	2.02	0.09	1.14	0.01	1.80	0.09	3.04	0.12	0.93	0.05	2.29	0.06	1.97	0.32
	2	1.44	0.06	0.70	0.03	3.30	0.25	0.61	0.06	1.10	0.11	2.63	0.13	2.06	0.02	1.02	0.03	1.05	0.17
-20	1	1.87	0.03	2.32	0.18	2.49	0.07	1.35	0.13	2.29	0.15	3.11	0.07	1.39	0.12	2.57	0.02	3.42	0.07
	2	0.91	0.63	0.64	0.28	3.81	0.15	0.99	0.04	1.64	0.14	2.43	0.06	1.70	0.02	1.62	0.12	1.23	0.08
-30	1	2.18	0.15	3.40	0.05	2.35	1.41	1.89	0.06	2.63	0.08	3.54	0.08	1.80	0.01	2.95	0.14	3.87	0.90
	2	1.57	0.12	1.16	0.12	5.29	0.07	1.34	0.73	1.95	0.11	3.27	0.33	2.35	0.02	1.91	0.02	2.14	0.16
-40	1	2.25	0.14	2.82	0.20	3.84	0.06	2.01	0.11	3.49	0.06	4.74	0.11	2.06	0.05	3.91	0.32	5.20	0.19
	2	3.41	0.10	1.59	0.10	7.25	0.18	1.49	0.06	1.80	0.14	5.37	0.13	2.52	0.04	1.92	0.10	2.26	0.69
-50	1	2.34	0.30	4.00	0.17	2.93	0.06	0.95	0.61	2.88	0.02	3.53	0.08	1.88	0.10	3.28	0.08	4.89	0.11
	2	2.39	0.57	1.08	0.23	5.19	0.12	1.24	0.09	2.29	0.09	4.61	0.28	2.23	0.08	1.79	0.04	2.25	0.04
-60	1	2.05	0.08	4.00	0.04	5.01	0.13	3.89	0.12	2.93	0.14	3.96	0.04	2.00	0.04	3.54	0.23	3.55	0.85
	2	1.63	0.31	0.82	0.02	4.96	0.19	1.44	0.05	2.23	0.04	4.76	0.13	3.12	0.09	2.37	0.13	2.14	0.02
-70	1	2.72	0.15	5.76	0.17	3.20	0.24	1.50	0.02	4.24	0.08	4.26	0.32	1.84	0.02	3.91	0.11	5.59	0.46
	2	2.04	0.07	1.32	0.81	3.63	2.42	1.27	1.20	2.41	0.27	3.96	0.15	3.28	0.06	2.00	0.03	2.28	0.08
-80	1	2.81	0.14	5.68	0.28	4.22	0.44	1.65	0.09	2.71	0.19	4.39	0.17	2.63	0.13	3.67	0.08	5.39	0.11
	2	1.28	0.50	1.57	0.07	5.92	0.12	1.71	0.09	2.13	0.13	4.86	0.17	3.91	0.07	2.36	0.08	2.10	0.05
1-way ANOVA		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p = 0.0307$		$p = 0.0341$		$p > 0.05$		$p > 0.05$	

a) n=3.

b) All  $p$ -values are for analyses without -10 °C.

2-way ANOVA: temperature:  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ .



Table 4. TBARS in cod fillets.

Temperature (°C)		Frozen storage (months)																							
		1		2		3		4		5		6		8		10		12		15		18			
		Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
-10 <sup>b</sup>	1	5.98	0.41	11.51	0.18	2.77	0.19	4.53	0.00	5.02	0.08	14.20	2.13	1.11	0.15	14.25	0.48	-0.03	0.15	2.51	0.37	2.48	0.00		
	2	2.29	0.24	4.71	0.24	2.78	0.42	3.26	0.80	0.66	0.38	1.22	0.08	1.27	0.00	38.80	0.40	0.08	0.32	1.64	0.38	3.10	0.08		
-20	1	3.33	0.16	2.97	0.24	3.35	0.15	2.56	0.00	0.75	0.00	1.82	0.35	0.13	0.08	0.71	0.08	-0.27	0.16	1.74	0.00	2.80	0.71		
	2	3.46	0.79	1.75	0.45	3.15	0.00	2.19	0.26	1.36	0.15	1.07	0.31	1.38	0.00	0.54	0.16	-0.20	0.38	2.03	0.37	0.64	0.32		
-30	1	4.20	0.38	3.25	0.40	4.55	0.08	2.58	0.00	1.70	0.00	1.39	0.07	0.39	0.00	1.56	0.48	0.32	0.67	4.06	0.81	5.05	0.24		
	2	3.46	0.08	2.13	0.00	3.90	0.00	2.98	0.00	1.33	0.08	1.56	0.16	1.18	0.00	2.36	0.55	0.13	0.08	3.54	1.43	2.28	0.62		
-40	1	3.79	0.08	1.46	0.08	3.74	0.00	2.20	0.27	2.08	0.00	1.62	0.00	0.63	0.00	1.16	0.08	0.92	0.00	3.57	1.16	3.95	0.16		
	2	4.23	0.09	3.55	0.16	4.05	0.00	2.67	0.40	1.97	0.00	1.24	0.00	2.18	0.08	0.71	0.08	0.00	0.00	3.44	1.59	2.53	0.00		
-50	1	2.85	0.47	3.10	0.08	4.42	0.67	2.56	0.00	1.05	0.00	1.46	0.39	1.31	0.00	0.98	0.32	2.48	0.48	2.18	0.53	7.19	0.00		
	2	2.59	0.16	4.00	0.08	4.55	0.32	3.51	0.00	1.26	0.00	2.54	0.32	3.23	0.14	1.72	0.08	1.65	0.38	3.17	1.09	5.37	0.16		
-60	1	4.10	0.23	3.36	0.00	4.26	0.69	2.80	0.28	1.46	0.08	1.70	0.08	1.31	0.08	1.54	0.38	0.86	0.32	1.98	0.15	5.43	0.15		
	2	2.65	0.08	1.40	0.00	2.89	0.00	4.84	0.00	1.37	0.07	1.84	0.00	4.24	0.00	1.60	0.40	0.36	0.08	2.37	1.47	3.51	0.39		
-70	1	4.82	0.06	3.16	0.08	3.27	0.24	2.59	0.00	1.31	0.00	1.85	0.26	0.40	0.00	2.10	0.00	1.44	0.22	3.81	1.56	0.98	0.16		
	2	3.11	0.39	1.30	0.16	3.05	0.15	2.20	0.00	0.80	0.00	1.46	0.16	2.18	0.00	1.12	0.55	0.24	0.08	2.34	0.22	4.05	0.00		
-80	1	5.23	0.16	2.33	0.00	3.57	0.00	2.96	0.03	1.05	0.00	1.52	0.16	0.07	0.00	1.61	0.15	1.51	0.08	5.04	0.15	4.78	0.08		
	2	2.56	0.08	1.38	0.00	4.02	0.15	3.93	0.54	0.99	0.00	1.02	0.08	1.24	0.15	0.74	0.00	2.84	0.00	1.80	0.00	2.42	0.08		
1-way ANOVA		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$			

a) n=2.

b) All  $p$ -values are for analyses without -10 °C.

2-way ANOVA: temperature:  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ .



## Paper II

---

Effect of frozen storage temperature on quality-related changes in rainbow trout (*Oncorhynchus mykiss*) and yellowfin tuna (*Thunnus albacares*).

Burgaard and Jørgensen (2010).

Accepted for publication in *Journal of Aquatic Food Product Technology*.

---

**Effect of frozen storage temperature on quality-related changes in rainbow trout (*Oncorhynchus mykiss*) and yellowfin tuna (*Thunnus albacares*).**

**Keywords:** Freezing, Fish, Quality indicators, ULT

**Running title:** Temperature effect on frozen trout and tuna quality

***ABSTRACT***

Rainbow trout (*Oncorhynchus mykiss*) and yellowfin tuna (*Thunnus albacares*) were stored at various temperatures from -10 to -80 °C for one to 18 months (rainbow trout) or two and three months (tuna). After each storage period, a number of quality-related measures were determined: drip loss, water holding capacity, low field NMR spin-spin relaxation, colour ( $L^*$ ,  $a^*$ ,  $b^*$ ), amount of thiobarbituric acid reactive substances and sarcoplasmic reticulum  $Ca^{2+}$ -ATPase and lysosomal Cathepsin D activities.

The results indicate that -30 °C seems to be sufficiently low for maintenance of the quality when storing tuna and rainbow trout for three and 18 months, respectively.

## **INTRODUCTION**

Fresh fish products are often regarded as superior to preserved products, but if fish is frozen immediately after catch and processing, stored at low, non-fluctuating temperatures and thawed in the best way according to its rigor-state, the quality can be as good as or better than fresh fish stored for several days at 0 °C (Cappeln et al., 1999; Nielsen and Jessen, 2007). Sørensen et al. (1995) term this 'fresh frozen products' and expand the phrase 'fresh' to include lightly salted, thawed, partly cooked or rehydrated fish as long as the consumer have a good sensory experience and perceive the product as fresh. Fatty species, such as trout, are stabile during frozen storage and are still suitable for consumption after 18 months, though only of high quality up to six months, assuming proper handling and packaging in an oxygen-free atmosphere (Nielsen and Jessen, 2007).

Factors that affect the quality of fish during frozen storage are quality of the raw material initially, storage conditions before freezing (e.g. time on ice), freezing rate, frozen storage temperature and time, temperature fluctuations, thawing procedure and prevention against oxidation (light and oxygen). Quality deteriorating processes can occur at all points of fish processing and the most common are physical alterations in the texture caused by large ice crystals (protein denaturation), freezer burn, lipid oxidation, protein oxidation and discolorations (Sikorski et al., 1976; Sikorski, 1978; Cappeln et al., 1999; Careche et al., 1999; Herrera et al., 2001; Leelapongwattana et al., 2005).

During freezing of fish muscle the crystallisation of water results in increased concentrations of salts and enzymes, thereby causing myofibrillar proteins to denature and aggregate. At -10 °C aggregation of trout myosin has been shown to be higher than at 0, -20 and -30 °C (Buttkus, 1970) and the rates of lipid and protein oxidation are also increased compared to unfrozen muscle despite the lower temperature. Therefore fast freezing is considered important in order to pass this critical temperature interval as fast as possible (Buttkus, 1970; Sikorski and Kolakowska, 1994). In addition, fast freezing results in the formation of many small ice crystals, whereas slow freezing may lead to formation of fewer and larger ice crystals. These are typically located extra cellular and as they are formed they can dehydrate the cells to an extent, that they remain unfrozen,

because of concentration of intra cellular solutions. Thereby reaction rates are increased further until falling temperature and increasing viscosity slows down the reaction rates again (Love, 1968; Wolfe and Bryant, 2001). Moreover, the formation of large ice crystals results in physical damage to structural proteins and cell membranes since extra cellular ice occupy far more space than the original extra cellular space and thus compresses the dehydrated cells (Love, 1968).

Numerous studies have focused on quality related processes occurring in the temperature interval between 0 and -30 °C and it is well known that storage at -20 °C or higher is insufficient to keeping a high quality. The effect of low temperatures, i.e. below -30 °C, is primarily studied in relation to tuna quality, especially colour (Watabe and Hashimoto, 1986; Chow et al., 1988), though, or with the use of a single temperature (-40 or -80 °C) or reference only.

In the present study, we conducted a comprehensive investigation on the relation between loss of quality, storage time and storage temperature in rainbow trout and tuna in the interval between -10 and -80 °C and storage times up to 18 months for trout and three months for tuna. Storage at -10 °C was included in order to have a ‘negative’ reference, because storage of fish at this temperature is known to cause extensive quality loss. The quality-related properties measured were: drip loss, water holding capacity, colour, lipid oxidation expressed as content of thiobarbituric acid-reactive substances (TBARS) and low-field nuclear magnetic resonance (NMR) spectroscopy, which gives information about the distribution of water in the muscle. In rainbow trout, the activities of the enzymes Sarcoplasmic Reticulum  $\text{Ca}^{2+}$ -ATPase (EC 3.6.1.38) and Cathepsin D (EC 3.4.23.5), were used as indicators for the integrity of the sarcoplasmic reticulum membrane and the lysosomes, respectively.

## **EXPERIMENTAL**

### ***Experimental design***

Rainbow trout (*Oncorhynchus mykiss*) for the experiments were obtained (November 2006) from a farm in Southern Denmark, slaughtered and bled by gill cutting on-site and transported on ice to the laboratory, where they were stored on ice for two days

until *rigor mortis* had passed. One 13.4 kg yellowfin tuna (*Thunnus albacares*) was bought from Vagn P. Fisk (Denmark), filleted, cut in 14 pieces of 30-40 g each, vacuum packed (12 mbar), frozen in a blast freezer at -40 °C and stored at -10, -20, -30, -40, -50, -60 and -80 °C for two or three months. The storage temperatures were stable with fluctuations within a few degrees. 22 trout (4-5 kg each) were filleted by hand on day three after slaughtering. The loin parts of the fillets were cut in four pieces each. These were further divided into a total of 40 small pieces of approximately 5.3·3 cm with a mean weight of 35 g (5 samples for different analyses stored at each of eight temperatures) and vacuum packed in plastic bags (NEN 40/LLDPE 75, Amcor Flexible A/S, Horsens Denmark) with an oxygen transmission of 6 cm<sup>3</sup>/m<sup>2</sup>/atm/24 h. After packaging, the 40 samples from each fish were randomly distributed to eight different freezers at temperatures between -10 and -80 °C (10 °C intervals) where they were frozen in still air at the storage temperature. The fish were freeze stored from one to 18 months (1, 2, 3, 4, 5, 6, 8, 10, 12, 15 and 18). In order to investigate the specific effect of different storage temperatures statistically, a paired experimental design was used. The 40 pieces from each fish were stored at eight different temperatures but only for one storage period, and for each storage period, two different trout were examined. Thus, all samples stored at the eight different temperatures for a certain time originated from the same two trout.

### *Analyses*

After each storage period, the rainbow trout and tuna were thawed in a 2 °C cold store overnight (samples for drip loss, water content, water holding capacity and NMR) or in 8 °C water (samples for the other analyses) and the following analyses were performed: low-field NMR- measurements, thiobarbituric acid reactive substances (TBARS), drip loss, water holding capacity, water content, colour measurements and activity of Cathepsin D and Ca<sup>2+</sup>-ATPase. Lipid content and fatty acid composition were measured on samples stored at -80 °C. For some of the analyses, fillet was minced for 5 s in a meat mincer (Foss Tecator Knifetec 1095 Sample Mill, Hillerød, Denmark). All analyses were made in duplicate unless otherwise indicated.



### ***Drip loss, water content and water holding capacity***

Filletts were weighed before and after thawing, and the drip loss was measured as the loss in mass divided by the initial mass of the fillet. Before weighing, excess drip from the surface of the thawed fillets was wiped off using a paper towel.

The water content was measured as the loss in mass after drying 2 g sample overnight at 105 °C divided by the original sample mass.

The water holding capacity was defined as the capacity of the minced muscle to hold its own cellular fluid when centrifuged (Eide et al., 1982). Portions of carefully mixed mince (2 g) were weighted into cylindrical tubes whose bottom was a filter allowing liquid but not solid material to pass. The tubes were then placed in 50 ml centrifuge tubes equipped with approximately 40 g glass marbles to provide space for the filtrate. Centrifugation was performed at 10 °C for 5 min at 1500 g in a Sigma 4K15 centrifuge equipped with a 12166 rotor. The water holding capacity was expressed as the mass of water remaining in the muscle sample after centrifugation divided by the mass of water in the original sample.

### ***Nuclear magnetic resonance***

The NMR-measurements were performed on portions of 2-3 g mince that were weighted into small cylindrical glass tubes fitting in the 18 mm NMR sample tubes. The samples were equilibrated for 30 min in an 8 °C water bath before measurements. Low-field relaxation measurements were performed on a Maran Benchtop Pulsed NMR analyser (Resonance Instruments, Witney, UK) operating at 23.2 MHz and equipped with an 18 mm variable temperature probe head. Transverse relaxation was measured at 8 °C as described by Jensen et al. (2002) using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. The 180°-pulse spacing ( $2\tau$ ) was 400  $\mu$ s and the number of data points acquired was 1024, spaced by  $4\tau$  (even echoes sampled).

### ***Enzyme activity***

Homogenates for determination of  $\text{Ca}^{2+}$ -ATPase and Cathepsin D activity were prepared from 3.0 g muscle cut into small pieces in 24 ml cold 10 mM Tris-HCl buffer (pH 7.0) containing 0.25 M sucrose. A Potter-Elvehjem apparatus was used to

homogenise the suspension by 10 up and down strokes with a Teflon pestle at 750 rpm. Afterwards the homogenate was centrifuged at 1000 g (4 °C) for 15 min and the supernatant was kept on ice or frozen at -80 °C until use.

The activity of  $\text{Ca}^{2+}$ -ATPase was measured using the method described by Godiksen and Jessen (2001) based on the work of Simonides and Hardeveld (1990). The activity of  $\text{Ca}^{2+}$ -ATPase was defined as the difference between liberated inorganic phosphate from ATP in the presence (total activity) and absence (basal activity) of free  $\text{Ca}^{2+}$ .

Cathepsin D activity in the homogenates was assayed using a slightly modified method of Yasuda et al. (1999), based on the fluorescence of the (7-methoxycoumarin-4-yl)acetyl (MOCAC) moiety which is splitted from the synthetic substrate MOCAC-Gly-Lys-Pro-Ile-Leu-Phe~Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH<sub>2</sub> catalysed by Cathepsin D. The substrate was prepared from 1.0 mg MOCAC-Gly-Lys-Pro-Ile-Leu-Phe~Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH<sub>2</sub> dissolved in 200  $\mu\text{l}$  acetate buffer (50 mM sodium acetate, pH 4.0) and 369  $\mu\text{l}$  dimethylsulfoxid (DMSO) and thereafter diluted 4 times in the same acetate buffer. The final concentration of DMSO in the assay was 1.28% and the substrate concentration 200  $\mu\text{M}$ . 80  $\mu\text{l}$  acetate buffer with 1 mM EDTA was added to the wells of a black assay plate (Corning Incorporated, NY) and heated for 15 min in a fluorescence spectrophotometer (Shimadzu UV-160A) at 35 °C. 10  $\mu\text{l}$  of sample solution (supernatant) and 10  $\mu\text{l}$  of substrate were added and the fluorescence of the cut substrate measured at an emission wavelength of 393 nm with excitation at 328 nm for 10 min. The slope of the curve fluorescence *versus* time was used as a measure of the activity of the enzyme.

#### ***Thiobarbituric acid-reactive substances (TBARS)***

TBARS was determined according to the method of Vyncke (1970) with the modification that the reaction was done at 90 °C instead of in boiling water.

#### ***Colour***

Colour parameters (CIE 1976, L\*, a\*, b\* colour space) were measured by a Minolta Chroma Meter CR-200 (Konica Minolta Sensing Inc., Japan). The values reported are average of triplicate measurements at two or three different measuring points on the flesh side of the fillet depending on its size.

### ***Lipid content and fatty acid composition***

The lipid content of the trout and tuna samples was determined by the method of Bligh and Dyer (1959) slightly modified by reducing solvent volumes. Fatty acid methyl esters were prepared using an AOCS official method (Ce 2-66, 1998) and analysed by gaschromatography, also using an official AOCS method (Ce 1 b-89, 1998). A HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an Omegawax 320 column (30m × 3.2mm, 0.25 µm; Supelco, Bellefonte, PA, USA) and a flame ionization detector (Hewlett-Packard, Palo Alto, CA, USA) was used for the analyses. The fatty acids were identified by comparison with chromatograms of standards.

### ***Data analysis***

Univariate one- and two-way analysis of variance (ANOVA) were used to look for possible differences in quality parameters due to temperature or time. According to the experimental design, repeated measures ANOVA was used between groups at different temperatures and a Bonferroni post test was used to compare all pairs of columns. Test for linear trend was made using regression on the temperature variable. The multivariate analysis methods principal component analysis (PCA) and partial least squares (PLS) regression were applied to the NMR relaxation curves with data from samples as rows in the *X*-matrix and (for the PLS regression) water holding capacity as *y*. Each column in the data matrix was mean-centred. The multivariate models were cross-validated using 2 segments, each with 88 members (samples from 11 different fish, stored for 11 different periods in each segment).

## RESULTS AND DISCUSSION

The average lipid content of the 22 trout was  $8.2 \pm 1.3$  % and of the tuna  $0.9 \pm 0.2$  %, the water content of trout was  $72.7 \pm 0.1$  % and of tuna  $72.9 \pm 0.4$  %. There were no significant differences in the polyunsaturated fatty acid composition of the 22 trout (1-way ANOVA,  $p \gg 0.05$ ).

### Drip loss and water holding capacity

The drip loss of trout was highest for samples stored at high temperatures and decreased with decreasing temperature (Test for linear trend  $p < 0.0001$ , Table 1) and generally samples stored at  $-10$  and  $-20$  °C had higher drip losses than samples stored at lower temperatures. The decrease in the WHC of trout during frozen storage was very similar to that seen in a similar experiment with cod (Burgaard & Jørgensen, *submitted*).

Samples stored at  $-10$  °C had a lower WHC than samples at all other temperatures after all storage times except one and three months (Table 2). After 12 months also samples at  $-20$  generally had reduced WHC compared to samples stored at lower temperatures, but no differences in WHC were seen between samples stored from  $-30$  to  $-80$  °C. This was also observed in cod, though reduced WHC in samples stored at  $-20$  °C compared to lower temperatures was already observed after 5 months (Burgaard & Jørgensen, *submitted*). Higher drip losses in cod frozen and stored at  $-10$  and  $-20$  °C compared to  $-40$  to  $-70$  °C were also reported by Mørkøre and Lilleholt (2007), but in their study the cod were frozen at different rates and only stored for 10-11 days, so freezing temperature probably had a more pronounced effect than storage temperature.

According to Mackie (1993), reduced WHC is primarily due to denaturation/aggregation of actin and in particular myosin. These are the main contractile proteins responsible for functional properties and their denaturation and aggregation is typically caused by ice crystal growth, increased ionic strength due to water crystallisation and protein and lipid oxidation (Sikorski et al., 1976; Shenouda, 1980; Mackie, 1993; Nott et al., 1999).

The drip loss and WHC of tuna were measured after two and three months (Table 3). The drip loss was higher for samples stored at  $-10$  compared to  $-20$  to  $-60$  °C, but there were no differences between samples stored at other temperatures or between samples

stored for two and three months. After both storage times there was an increase in WHC with decreasing temperature (linear trend: 2 months,  $p < 0.001$ ; 3 months,  $p = 0.002$ ), but no significant differences in means between different storage temperatures (1-way ANOVA). Surprisingly, samples stored for three months had a significantly higher WHC compared to two months, and that was not related to differences in drip loss. All WHC's were, however, very high (over 89 %).

### ***NMR***

The NMR relaxation curves were used as x-variables in a PCA. Samples stored at -10 °C were different from all other samples and when removing these from the data set also samples stored at -20 °C were different (Fig. 1). Samples stored at -20 °C for 12-18 months were grouped away from samples stored at -30 to -80 °C, whereas most of the samples stored at -20 °C for 1-10 months were in the area between the two groups. This is in agreement with the results of the WHC of trout. It was not possible to see any differences between samples stored at temperatures from -30 to -80 °C. In a study by Jensen et al. (2003) a higher proportion of slowly relaxing (loosely bound) water in cod samples stored at -20 for at least three months separated these from samples stored at -30 °C. A similar relationship was shown by Steen and Lambelet (1997), who were able to separate cod mince stored at -10 °C for 4 months from samples stored at -20 and -70 °C, also based on a larger proportion of slowly relaxing water in samples stored at the highest temperature. In this study NMR relaxation curves were normalised according to total signal amplitude, so differences between samples are due to differences in water distribution and not total water content. Samples stored at -20 °C did not begin to differ from samples stored at -30 to -80 °C until after 12 months of frozen storage and samples stored at -30 °C was not different (according to the NMR measurements) from samples stored at lower temperatures at any time. This was expected as trout is more robust to frozen storage compared to cod and according to Nielsen and Jessen (2007) trout and other fatty species have a storage life (consumer acceptability) of at least 18 months of frozen storage at -30 °C. Water distribution and binding are affected by textural changes in the muscle proteins and NMR  $T_2$ -values are shown to correlate with several analytical methods (instrumental and sensory) (Steen and Lambelet, 1997), therefore there seem to be a higher degree of protein denaturation

in samples stored at -10 and, after 12 months of frozen storage, also -20 °C compared to storage at lower temperatures.

For samples stored at -30 to -80 °C it was not possible to see any differences caused by storage temperature using NMR spectroscopy.

The WHC of the samples were predicted using the NMR-relaxation curves as  $X$ -matrix and the measured WHC as  $y$ -vector in a PLS regression. Predicted and measured WHC correlated well ( $r = 0.84$ ,  $n=2$ ) considering an error of approximately 2.9 % in the WHC analysis. High correlations between WHC and relaxation data have also been reported for cod by Andersen and Jørgensen (2004) and Jepsen et al. (1999).

There were no systematic differences between relaxation curves from NMR-measurements on tuna samples stored at different temperatures for two to three months.

### ***Ca<sup>2+</sup>-ATPase activity***

Trout samples stored at -10 and -20 °C generally had significantly lower Ca<sup>2+</sup>-ATPase activities than samples stored at -30 °C or lower (1-way ANOVA) during 18 months of frozen storage (Fig. 2). This is in agreement with the results of Godiksen et al. (2003), who were able to distinguish between cod stored for five months at -30 (highest SR Ca<sup>2+</sup>-ATPase activity) and -20 °C. Hsu et al. (1993) reported a more rapid decrease in actomyosin Ca<sup>2+</sup>-ATPase activity in Pacific Whiting stored at -8 °C compared to samples stored at -20, -34 and -50 °C. However, del Mazo et al, (1999) did not observe any significant differences between actomyosin Ca<sup>2+</sup>-ATPase activity in hake fillets stored at -20 and -30 °C for 49 weeks. The activity decreased significantly at both temperatures and no activity was detected from 36 weeks onwards, which was ascribed to denaturation of actomyosin. In the interval from four to 12 months Ca<sup>2+</sup>-ATPase activity in trout samples stored at temperatures between -30 and -80 °C increased dramatically. This was also observed in a previous study on cod (Burgaard & Jørgensen, *submitted*), where the activation was seen in the interval from two to 10 months of frozen storage. Inoue et al. (1992) reported an increased Ca<sup>2+</sup>-ATPase activity of frozen carp myosin during the first 12 hours of frozen storage for samples stored at -15 and -20 °C, while the activity of samples stored at -5 to -13 °C decreased. Activation of SR Ca<sup>2+</sup>-ATPase activity as a result of freezing was also reported by Godiksen et al. (2003),

who suggested that the freeze-induced activation of the enzyme was due to changed interactions between  $\text{Ca}^{2+}$ -ATPase and the surrounding membrane lipids.

### ***Cathepsin D activity***

Storage temperature did not seem to affect Cathepsin D activity in trout (Table 4). There were, however, large variations in activity over time; probably due to variations between different individuals. According to Nilsson and Ekstrand (1995) frozen storage temperature affect lysosomal membrane integrity resulting in increased lysosomal enzyme leakage and thereby increased activity of  $\beta$ -N-acetylglucosaminidase for trout samples stored at  $-18\text{ }^{\circ}\text{C}$  compared to  $-40\text{ }^{\circ}\text{C}$ . Cathepsin D could be activated the same way, while decrease in activity over time can be caused by protein denaturation. These two ‘opposite’ reactions might also explain the unclear results, which are very difficult to interpret in relation to the effect of temperature on protein denaturation/oxidation and membrane integrity

### ***TBARS***

The change in TBARS for trout samples at different temperatures over time is illustrated in Table 5. The TBARS content increased with time for samples stored at  $-10$  and  $-20\text{ }^{\circ}\text{C}$  and were significantly higher compared to samples stored at or below  $-30\text{ }^{\circ}\text{C}$  ( $p<0.001$  for all) during the time of the experiment. Similar results for saithe were reported by Dulavik et al. (1998) and Aubourg et al. (2004) observed higher peroxide value (PV) and TBARS content in horse mackerel fillets stored at  $-20$  compared to  $-80\text{ }^{\circ}\text{C}$ . Rodríguez et al. (2007) only observed a low degree of lipid oxidation (PV, TBARS) in farmed coho salmon stored at  $-20\text{ }^{\circ}\text{C}$  and did not observe any significant increase in TBARS content until after 15 months of frozen storage (TBARS-value  $0.54\text{ mg/kg}$ ). This value corresponds approximately to the level of  $8.3\text{ }\mu\text{mol/kg}$  sample for trout stored at  $-20\text{ }^{\circ}\text{C}$  for eight months in our experiment, and the authors attributed the oxidative stability to stable endogenous antioxidants. The level of TBARS in our study did, however, not increase much from eight to 18 months. The samples stored at  $-10$  and  $-20\text{ }^{\circ}\text{C}$  in our experiment were placed directly in the freezer at these temperatures, therefore experiencing a slow cooling rate. According to Geromel and Montgomery (1980), a slow rate of freezing can cause lipase to be released from the lysosomes in

rainbow trout and thereby enhance lipid oxidation, whereas intermediate or fast freezing rates do not result in significant increase in lipase release.

In tuna there was an obvious relationship between increased lipid oxidation and increased storage temperature (Table 6). Samples stored at -10 °C had significantly higher TBA-values than samples stored at lower temperatures and samples stored at -20 and -40 °C higher than -50 to -80 °C. Samples stored at -30 °C had a significantly higher TBA-value compared to samples stored at -80 °C and there were no differences in TBA-value for samples stored at -50 to -80 °C.

### ***Colour***

Colour measurements were performed using the CIE 1976, L\*, a\*, b\* colour space. Values for L\*, a\* and b\* were analysed using PCA supplemented by 1-way ANOVA. Possible differences in colour due to storage temperature and time were blurred by large individual variations in the colour of the trout samples (results not shown). Colour measurements were performed on intact as well as minced tuna samples. The values for L\*, a\* and b\* were analysed using 1- and 2-way ANOVA. The a\*-values for whole and minced tuna fillets increased with decreasing storage temperature (Test for linear trend  $p = 0.046$  (whole) and  $p < 0.0001$  (minced), Fig. 3), while the L\*-values for whole and minced samples and the b\*-values for whole samples decreased with decreasing storage temperature (Test for linear trend  $p = 0.0125$  (L\*, whole),  $p = 0.0131$  (L\*, minced) and  $p = 0.03$  (b\*, whole)). The b\*-values for minced samples did not differ with storage temperature (Test for linear trend,  $p > 0.05$  for both).

Tuna samples stored at -80 °C were darker than samples stored at -10 and -20 °C and -60 were darker than -10 °C after three months of frozen storage (2-way ANOVA). Samples stored at -30 to -80 °C were significantly more red (higher a\*) after three months of frozen storage and samples stored at -40 to -80 °C also after two months compared to samples stored at -10 and -20 °C (2-way ANOVA). This is in fair agreement with Watabe and Hashimoto (1986), who measured colour in tuna meat using the ratio of met-myoglobin to total myoglobin. They showed that this ratio was at the same level for tuna samples stored at -20, -40, -60 and -80 °C for the first three months of frozen storage, after which the ratio for samples stored at -20 °C increased



(became more brown) until the end of the experiment (12 months) and samples stored at -40 to -80 °C stayed unchanged. Chow et al. (1988) also demonstrated that discoloration of tuna meat proceeded steadily during storage at -20 °C and was accelerated upon thawing, whereas storage below -30 °C effectively prevented accelerated discoloration upon thawing.

### ***CONCLUSION***

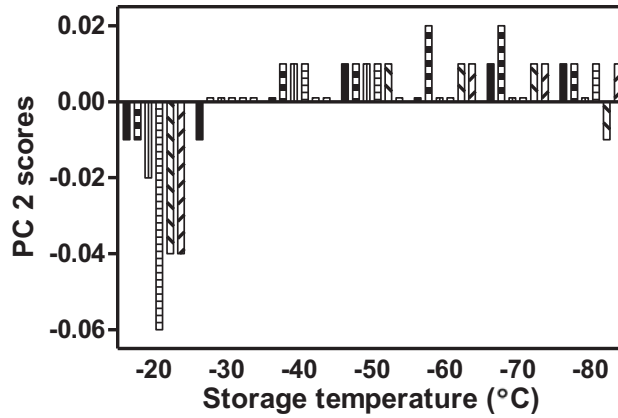
A temperature of -30 °C seems to be sufficiently low for storage of trout up to 12 months and tuna up to at least three months. No gain in preservation of quality as measured by a wide range of indicators was obtained by using lower temperatures.

## REFERENCES

- Andersen, C.M. and Jørgensen, B.M. 2004. *J. Aquat. Food Prod. Technol.* 13(1): 13-23.
- AOCS. 1998. Official Method Ce 1 b-89. Fatty Acid Composition by GLC. Marine Oils. AOCS, Champaign, IL.
- AOCS. 1998. Official Method Ce 2-66. Preparation of methyl esters of fatty acids. AOCS, Champaign, IL.
- Aubourg, S.P., Pineiro, C., and Gonzalez, M.J. 2004. *J. Am. Oil Chem. Soc.* 81(7): 671-678.
- Bligh, E.G. and Dyer, W.J. 1959. *Can. J. Biochem. Physiol.* 37: 911-917.
- Buttkus, H. 1970. *J. Food Sci.* 35: 558-562.
- Cappeln, G., Nielsen, J., and Jessen, F. 1999. *J. Sci. Food Agric.* 79(8): 1099-1104.
- Careche, M., Herrero, A.M., Rodriguez-Casado, A., del Mazo, M.L., and Carmona, P. 1999. *J. Agric. Food Chem.* 47(3): 952-959.
- Chow, C.J., Ochiai, Y., Watabe, S., and Hashimoto, K. 1988. *Bull. Jap. Soc. Sci. Fish.* 54(4): 639-648.
- del Mazo, M.L., Torrejon, P., Careche, M., and Tejada, M. 1999. *J. Agric. Food Chem.* 47(4): 1372-1377.
- Dulavik, B., Sorensen, N.K., Barstad, H., Horvli, O., and Olsen, R.L. 1998. *J. Food Lipids.* 5(3): 233-245.
- Eide, O., Børresen, T., and Strøm, T. 1982. *J. Food Sci.* 47: 347-349, 354.
- Geromel, E.J. and Montgomery, M.W. 1980. *J. Food Sci.* 45(3): 412.
- Godiksen, H., Hyldig, G., and Jessen, F. 2003. *J. Food Sci.* 68(8): 2579-2585.
- Godiksen, H. and Jessen, F. 2001. *J. Food Biochem.* 25(4): 343-358.
- Herrera, J.J., Pastoriza, L., and Sampedro, G. 2001. *J. Sci. Food Agric.* 81(4): 377-384.

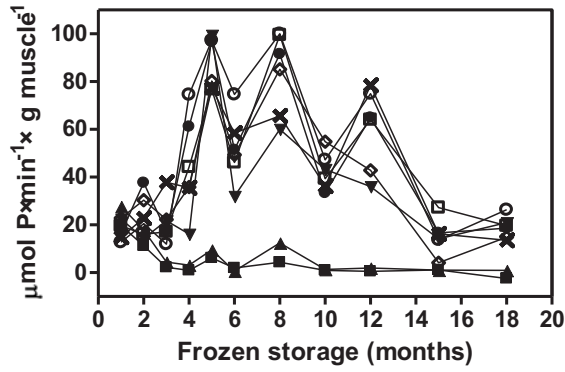
- Hsu, C.K., Kolbe, E., Morrissey, M.T., and Chung, Y.C. 1993. *J. Food Sci.* 58(5): 1055-1056.
- Inoue, N., Takatori, K., Motoshige, T., and Shinano, H. 1992. *Jap. Soc. Sci. Fish.* 58(12): 2357-2360.
- Jensen, K.N., Guldager, H.S., and Jørgensen, B.M. 2002. *J. Aquat. Food Prod. Technol.* 11(3/4): 201-214.
- Jensen, K.N. and Jørgensen, B.M. 2003. *Lebensm.-Wiss. Technol.* 36(8): 807-812.
- Jepsen, S.M., Pedersen, H.T., and Engelsen, S.B. 1999. *J. Sci. Food Agric.* 79(13): 1793-1802.
- Leelapongwattana, K., Benjakul, S., Visessanguan, W., and Howell, N.K. 2005. *Food Chem.* 90(1-2): 141-150.
- Love, R.M. 1968. Ice Formation in Frozen Muscle. In: *Low Temperature Biology of Foodstuffs*. Hawthorn, J. and Rolfe, E. J. (Ed.) Pergamon Press, Oxford, 105-124.
- Mackie, I.M. 1993. *Food Rev. Int.* 9(4): 575-610.
- Mørkøre, T. and Lilleholt, R. 2007. *J. Texture Stud.* 38(4): 457-472.
- Nielsen, J. and Jessen, F. 2007. Quality of Frozen Fish. In: *Handbook of Meat, Poultry and Seafood Quality*. Nollet and L.M.L. (Ed.) Blackwell Publishing, 577-586.
- Nilsson, K. and Ekstrand, B. 1995. *J. Food Sci.* 60(3): 627.
- Nott, K.P., Evans, S.D., and Hall, L.D. 1999. *Magn. Reson. Imaging.* 17(3): 445-455.
- Rodriguez, A., Losada, V., Larrain, M.A., Quitral, V., Vinagre, J., and Aubourg, S.P. 2007. *J. Am. Oil Chem. Soc.* 84(8): 727-734.
- Shenouda, S.Y.K. 1980. *Adv. Food Res.* 26: 311.
- Sikorski, Z.E. 1978. *Int. J. Refrig.* 1(3): 173-180.
- Sikorski, Z.E. and Kolakowska, A. 1994. Changes in Proteins in Frozen Stored Fish. In: *Seafood Proteins*. Sikorski, Z. E., Pan, B. S. and Shahidi, F. (Ed.) Chapman and Hall, New York. pp. 99-112.

- Sikorski, Z.E., Kostuch, S., and Olley, J. 1976. *CRC Critical Reviews in Food Science and Nutrition*. 8(1): 97-129.
- Simonides, W.S. and Vanhardeveld, C. 1990. *Anal. Biochem.* 191(2): 321-331.
- Sørensen, N.K., Brataas, R., Nyvold, T.E. and Lauritzen, K. 1995. Influence of early processing (pre-rigor) on fish quality. In: *Seafood from producer to consumer, integrated approach to quality*. Luten, J. B., Børresen, T. and Oehlenschläger, J. (Ed.) Elsevier Science B.V., Amsterdam. pp. 253-263.
- Steen, C. and Lambelet, P. 1997. *J. Sci. Food Agric.* 75(2): 268-272.
- Vyncke, W. 1970. *Fette Seifen Anstrichmittel*. 72(12): 1084-1087.
- Watabe, S. and Hashimoto, K. 1986. *Food Rev. Int.* 2(3): 353-393.
- Wolfe, J. and Bryant, G. 2001. *Int. J. Refrig.* 24(5): 438-450.
- Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y., and Yamamoto, K. 1999. *J. Biochem.* 125: 1137-1143.



**Figure 1.** PC2-scores from a PCA on NMR relaxation measurements on rainbow trout fillets.

5% of the variation was accounted for by PC2. Symbols used: ■, 1 month; ▨, 6 months; ▩, 10 months; ▤, 12 months; ▥, 15 months; ▦, 18 months.

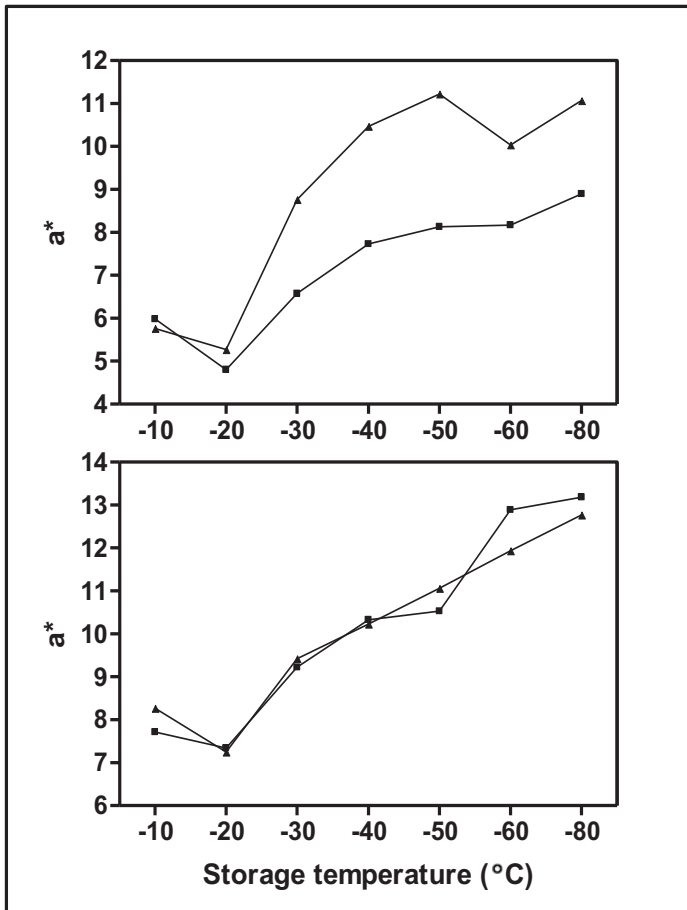


**Figure 2.** Effect of time and temperature on  $\text{Ca}^{2+}$ -ATPase activity in frozen-stored rainbow trout.

Symbols used: ■, -10; ▲, -20; ▼, -30; ●, -40; ×, -50; ◇, -60; ○, -70; □, -80 °C.

2-way ANOVA: temperature,  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ .

Samples at -10 °C were excluded from the 2-way ANOVA.  $n=2$  for each temperature.



**Figure 3.** a\*-values of whole (top) and minced (bottom) tuna fillets.

Symbols: ■, 2 months; ▲, 3 months.

Test for linear trend: whole fillet;  $p = 0.0046$ ; minced fillet;  $p < 0.0001$ .

Table 1. Drip loss of trout fillets.

Temperature (°C)	Fish	Frozen storage (months)										
		1	2	3	4	5	6	8	10	12	15	18
-10°	1	6.27	9.32	4.95	7.48	8.80	12.81	7.78	7.69	11.31	8.72	9.03
	2	11.20	9.39	3.61	11.19	7.31	9.45	11.35	11.02	5.63	3.96	6.25
-20	1	6.00	8.70	6.12	9.28	7.65	10.84	12.11	9.52	5.89	7.17	10.26
	2	8.86	8.77	5.01	9.36	7.14	5.27	6.06	8.77	10.31	8.48	6.60
-30	1	4.19	6.15	9.02	5.02	5.50	5.60	4.68	5.86	8.23	6.18	6.84
	2	7.68	0.39	5.85	5.53	5.19	7.44	6.06	7.95	9.29	6.18	7.92
-40	1	4.03	7.52	9.09	5.37	5.07	8.24	3.78	7.93	5.61	8.25	9.48
	2	6.69	6.42	6.46	11.04	6.59	6.40	5.57	7.75	7.56	3.72	4.94
-50	1	6.43	6.61	5.85	4.22	3.61	9.24	6.39	6.45	10.04	7.99	6.20
	2	5.93	6.89	6.61	8.89	5.88	4.16	6.90	9.71	8.96	5.41	4.50
-60	1	4.51	7.21	6.28	5.48	4.76	6.87	5.39	8.81	5.82	6.20	6.36
	2	5.79	7.12	3.46	5.32	5.20	6.29	4.87	7.58	6.60	7.13	4.54
-70	1	7.77	7.96	7.63	4.87	4.12	9.28	4.39	6.03	5.08	8.49	6.05
	2	6.53	5.62	4.04	4.91	5.38	4.88	6.18	6.69	8.32	6.43	4.82
-80	1	6.30	7.15	5.23	3.27	3.89	7.70	3.18	7.32	7.76	9.63	4.87
	2	7.15	6.17	5.41	7.94	6.32	6.31	6.42	6.48	6.27	6.04	2.16
1-way ANOVA		$p = 0.093$	$p = 0.127$	$p = 0.035$	$p = 0.069$	$p = 0.063$	$p = 0.055$	$p = 0.80$	$p = 0.52$	$p = 0.17$	$p = 0.13$	$p = 0.023$

a) All  $p$ -values are for analyses without -10 °C.

1-way ANOVA:  $p < 0.0001$ .

Linear trend:  $p < 0.0001$ .



Table 2. Water holding capacity of trout fillets.

Temperature (°C)		Frozen storage (months)																	
		1		2		3		4		5		6		8		10		12	
		Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
-10 <sup>b</sup>	1	94.26	0.89	85.12	4.24	94.89	0.06	75.09	2.52	87.22	15.94	76.87	3.44	72.97	5.20	67.08	0.71	67.84	3.69
	2	95.49	0.56	89.25	0.09	88.13	1.00	87.03	2.08	71.32	9.22	70.76	3.49	78.14	1.43	67.17	3.19	66.02	3.39
-20	1	96.19	0.24	93.55	0.83	98.05	0.23	95.10	0.17	95.09	0.33	92.49	1.37	96.63	1.26	89.59	0.24	89.04	1.34
	2	97.89	0.22	96.83	1.91	98.10	0.38	97.50	0.11	97.98	8.83	94.38	0.99	91.95	0.68	90.88	0.31	91.84	1.51
-30	1	96.12	1.10	93.79	0.85	97.98	0.43	96.59	1.32	96.65	3.88	95.84	0.12	94.95	0.74	92.40	0.61	97.18	0.01
	2	96.99	0.34	98.45	0.17	98.15	0.23	98.27	0.53	97.04	0.28	97.62	0.48	96.68	0.21	97.30	0.08	97.84	0.04
-40	1	97.42	0.58	96.34	0.19	98.84	0.13	97.59	0.27	92.11	0.52	96.56	0.55	95.63	1.22	97.94	0.47	97.79	0.24
	2	97.97	0.28	98.20	0.10	98.59	0.11	98.42	0.32	97.53	0.63	98.16	0.24	97.19	0.82	95.04	0.16	98.42	0.06
-50	1	97.11	0.42	97.16	0.41	97.97	0.28	97.16	0.40	94.82	0.22	96.35	0.13	97.95	0.73	95.82	0.23	97.81	0.57
	2	97.22	0.06	96.05	0.23	95.18	0.61	98.37	0.48	97.20	0.17	97.90	0.39	97.10	0.72	96.80	0.23	97.17	0.53
-60	1	97.80	0.27	97.74	0.62	103.01	6.22	98.08	0.32	92.87	7.12	96.98	0.59	97.07	0.72	90.81	1.29	98.32	0.18
	2	98.08	0.44	96.54	0.09	97.49	0.58	98.63	0.18	96.40	0.95	98.03	0.20	97.74	0.28	95.48	0.57	95.93	0.47
-70	1	97.45	0.04	97.52	0.52	98.72	0.20	97.55	0.34	96.04	5.39	97.86	0.64	97.76	0.26	92.29	0.31	97.85	0.62
	2	97.12	0.32	97.60	0.17	98.26	0.27	98.61	0.42	96.23	0.87	98.41	0.04	96.99	0.50	94.06	0.07	95.04	0.86
-80	1	97.29	0.10	98.02	0.06	98.61	0.18	91.58	0.87	97.91	0.32	95.55	0.54	95.51	0.34	96.78	0.39	98.25	0.18
	2	97.86	0.31	96.39	0.39	98.58	0.11	98.86	0.03	98.18	0.09	98.38	0.05	98.59	0.17	95.95	0.35	98.38	0.22
1-way ANOVA		$p = 0.19$		$p = 0.82$		$p = 0.48$		$p = 0.53$		$p = 0.35$		$p = 0.0012$		$p = 0.57$		$p = 0.12$		$p = 0.011$	
a) n=2.																		$p = 0.018$	$p = 0.0089$

b) All  $p$ -values are for analyses without -10 °C.

2-way ANOVA: temperature,  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ .

Table 3. Drip loss and WHC of tuna samples.

Temperature (°C)	Frozen storage (months)					
	Drip loss (%)		WHC (%)			
			2		3	
	2	3	Mean <sup>a</sup>	SD	Mean	SD
-10 <sup>b</sup>	9.8	9.7	90.96	1.41	94.98	0.21
-20	7.2	7.4	89.11	0.11	95.57	1.48
-30	5.8	6.2	93.20	1.03	96.84	0.94
-40	6.6	7.6	93.55	2.52	96.97	0.81
-50	6.0	7.3	90.93	0.96	98.18	0.17
-60	7.0	6.4	95.27	0.34	98.13	0.56
-80	7.4	8.3	96.25	0.76	97.35	0.13
1-way	$p > 0.05$		$p = 0.0083$		$p = 0.12$	
ANOVA						

a) n=1 sample; duplicate determination, n=2.

b) All  $p$  -values are for analyses without -10 °C.

2-way ANOVA (WHC): temperature,  $p < 0.0001$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ .

Table 4. Cathepsin D activity in trout fillets.

Temp. (°C)		Frozen storage (months)																							
		1		2		3		4		5		6		8		10		12		15		18			
		Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
-10 <sup>b</sup>	1	2.36	1.37	2.99	0.32	2.71	0.40	1.33	0.13	2.14	0.54	2.64	0.05	2.97	0.09	2.21	0.05	2.01	0.11	1.46	0.26	1.03	0.26		
	2	0.91	0.77	10.05	1.15	3.56	0.11	1.55	0.04	0.79	0.03	4.03	0.08	4.48	0.35	3.75	0.78	3.26	0.07	1.30	0.37	1.50	0.50		
-20	1	1.56	1.17	8.14	4.27	5.53	0.17	1.69	0.09	2.16	0.19	4.51	3.94	4.18	1.09	2.91	0.60	2.84	0.73	1.99	0.45	1.64	0.67		
	2	1.27	0.23	9.14	0.99	3.93	0.60	2.02	0.02	1.69	0.14	6.27	0.37	4.85	0.41	4.84	0.98	3.54	0.27	1.54	0.21	2.23	0.71		
-30	1	1.35	0.50	3.72	0.65	4.62	0.25	2.52	0.13	1.97	0.31	5.91	0.10	4.48	0.25	3.37	0.06	3.58	0.17	2.75	0.86	2.13	0.27		
	2	1.15	0.08	4.95	2.81	4.75	0.38	2.22	0.23	2.24	0.14	5.84	0.36	3.62	2.10	3.76	2.35	2.73	1.06	1.68	0.27	2.54	0.02		
-40	1	2.03	0.64	4.41	0.76	4.21	0.02	1.17	0.12	1.93	0.01	6.07	1.35	5.02	0.10	4.36	0.22	3.07	0.70	3.50	0.06	2.18	0.19		
	2	0.97	0.50	10.05	1.15	3.04	0.31	3.84	0.28	2.12	0.01	5.14	1.02	1.24	0.01	1.11	0.02	1.38	0.04	1.20	0.13	2.34	0.05		
-50	1	1.41	0.76	3.47	0.61	3.68	0.16	1.40	0.13	1.46	0.56	5.16	0.17	5.04	0.21	4.44	0.08	3.71	0.26	2.65	0.10	3.08	0.83		
	2	1.66	0.11	10.17	0.41	4.95	0.12	2.43	0.41	0.74	0.68	5.87	0.36	2.69	0.00	2.45	0.17	1.93	0.14	1.83	0.11	3.14	0.22		
-60	1	2.25	0.19	5.93	3.06	3.12	0.22	1.93	0.05	2.09	0.21	5.59	0.30	4.95	0.24	4.30	0.19	3.05	0.57	1.74	0.79	2.49	0.51		
	2	1.03	0.96	9.10	0.73	3.80	0.69	2.72	0.09	1.41	0.93	4.68	0.92	3.13	0.39	3.04	0.52	3.29	1.19	2.45	0.56	2.80	0.50		
-70	1	2.23	0.41	9.14	0.99	4.09	1.03	1.35	0.08	1.64	0.82	5.76	0.16	4.67	0.12	3.97	0.52	2.95	0.73	1.79	0.58	2.05	0.34		
	2	1.47	0.15	3.03	5.23	2.88	2.51	3.41	0.14	1.50	0.29	5.80	0.21	3.21	0.30	3.16	0.19	3.46	0.94	2.77	0.23	2.51	0.33		
-80	1	2.52	0.62	4.95	2.81	6.58	0.61	1.18	0.06	1.88	0.11	3.34	0.59	4.67	0.13	4.20	0.18	3.75	0.13	2.38	0.05	1.70	0.02		
	2	0.57	0.59	0.00	0.01	2.68	0.98	1.40	1.27	1.62	0.22	4.34	0.97	2.67	0.07	2.80	0.09	2.63	0.12	2.54	0.01	3.33	0.28		
1-way ANOVA		$p = 0.94$		$p = 0.64$		$p = 0.81$		$p = 0.68$		$p = 0.11$		$p = 0.22$		$p = 0.87$		$p = 0.97$		$p = 0.80$		$p = 0.98$		$p = 0.21$			

a) n=3.

b) All  $p$ -values are for analyses without -10 °C.

2-way ANOVA: temperature,  $p = 0.0011$ ; time,  $p < 0.0001$ ; interaction,  $p < 0.0001$ .

Table 5. TBARS in trout fillets.

Temperature (°C)		Frozen storage (months)																	
		1		2		4		6		8		10		12		15		18	
		Mean <sup>a</sup>	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
-10 <sup>b</sup>	1	8.64	7.69	7.43	1.19	10.84	0.14	16.69	0.31	21.04	1.15	21.00	0.56	24.43	0.16	15.93	0.08	34.82	0.16
	2	0.14	1.12	5.68	0.03	11.51	0.98	11.23	0.39	25.16	0.79	14.07	0.00	17.44	0.00	18.93	0.62	27.36	0.16
-20	1	4.36	1.07	4.42	0.27	4.26	0.27	3.64	0.08	9.28	0.13	8.42	0.55	10.35	0.23	8.18	0.15	10.19	0.40
	2	3.21	3.02	3.72	0.44	7.95	0.84	3.82	0.16	7.34	0.14	6.87	0.07	5.40	0.08	5.88	0.08	10.14	0.24
-30	1	15.00	9.79	2.15	0.61	4.15	0.16	1.66	0.08	4.52	0.00	3.51	0.00	1.70	0.08	1.82	0.15	4.33	0.08
	2	0.59	0.18	1.60	0.36	4.82	0.00	0.95	0.00	4.24	0.13	3.67	0.00	1.76	0.00	1.72	0.00	3.52	0.16
-40	1	1.66	1.69	1.65	0.32	2.35	0.02	1.34	0.08	3.25	0.00	2.91	0.08	1.15	0.00	-0.59	0.11	1.89	0.15
	2	0.96	0.69	0.26	0.08	2.24	0.70	0.34	0.23	3.03	0.00	1.74	0.00	1.09	0.00	0.08	0.00	1.83	0.00
-50	1	8.73	5.34	3.97	0.83	3.50	0.10	1.02	0.00	3.25	0.38	0.64	0.00	0.30	0.00	-0.20	0.23	1.56	0.16
	2	11.79	3.41	2.53	0.72	3.26	0.08	3.37	0.08	2.76	0.13	1.99	0.00	0.19	0.00	-0.69	0.00	1.22	0.16
-60	1	5.79	6.37	1.88	0.05	1.54	0.13	1.75	0.36	2.72	0.13	1.85	0.00	-0.19	0.08	-1.01	0.00	0.76	0.07
	2	1.79	0.57	1.23	0.04	1.47	0.00	3.73	0.16	3.11	0.53	1.76	0.00	0.13	0.08	-1.06	0.08	1.95	0.00
-70	1	2.33	0.38	4.46	0.87	2.82	0.17	0.85	0.16	3.01	0.13	1.76	0.00	0.08	0.00	-0.28	0.52	2.45	0.15
	2	-1.10	1.43	1.98	0.41	2.72	0.16	2.24	0.05	2.32	0.26	2.29	0.08	-0.14	0.00	-1.29	0.08	1.46	0.30
-80	1	2.15	0.97	2.47	0.28	2.46	0.16	0.89	0.08	2.73	0.00	1.30	0.00	0.02	0.40	-0.60	0.16	0.41	0.00
	2	7.43	0.12	2.61	0.51	1.97	0.31	0.97	0.07	2.43	0.40	1.84	0.00	0.09	0.00	-0.57	0.15	0.77	0.08
1-way ANOVA		p = 0.43		p = 0.017		p = 0.042		p = 0.13		p = 0.0002		p = 0.0011		p = 0.0080		p = 0.0002		p < 0.0001	

a) n=2.

b) All *p*-values are for analyses without -10 °C.

2-way ANOVA: temperature, *p* < 0.0001; time, *p* = 0.0003; interaction, *p* < 0.0001.

Table 6. TBARS in tuna fillets.

	Frozen storage (months)	
	Mean <sup>a</sup>	SD
-10 <sup>b</sup>	6.04	0.52
-20	3.75	0.05
-30	3.27	0.55
-40	3.99	0.16
-50	2.08	0.17
-60	2.21	0.33
-80	1.68	0.16
1-way ANOVA		$p = 0.0008$

a) n=1 sample; duplicate determination, n=2.

b) The  $p$  -value is for analysis without -10 °



## **Paper III**

---

Ice crystallisation point and unfrozen water in fish muscle.

Burgaard and Jørgensen (2010).

Manuscript

---

## **Ice crystallisation point and unfrozen water in fish muscle.**

Maria G. Burgaard and Bo M. Jørgensen

*National Food Institute, Division of Seafood Research, Technical University of Denmark, Søtofts Plads, Build. 221, DK-2800 Kgs.Lyngby, Denmark.*

### **Abstract**

The ice crystallisation point and the fraction of unfrozen water in tuna (*Thunnus albacares*), cod (*Gadus morhua*) and herring (*Clupea harengus*) muscle were determined by differential scanning calorimetry (DSC). The area of the ice melting curve after annealing at a temperature below the ice crystallisation point and subsequent heating was used to calculate the relative amount of unfrozen water in the fish muscle. The ice crystallisation point was defined as the lowest annealing temperature above which no melting peak could be detected. This point was around -15 °C showing a high degree of super-cooling in the species investigated. At temperatures below the ice crystallisation point, no significant difference in fraction of unfrozen water was found which is in opposition to common belief that the fraction of unfrozen water decreases gradually with decreased temperature. Previously frozen and thawed cod contained a significantly ( $p<0.001$ ) lower fraction of unfrozen water compared to fresh cod. The results indicate that proper frozen storage of fish requires congelation at a temperature lower than -15 °C.

### **Keywords**

DSC, super-cooling, muscle water, freezing point, frozen storage



## INTRODUCTION

The amount of water and its physical state in foods are important properties, influencing quality and shelf life. Muscle based foods typically consist of 70-80% water, which is distributed between different 'water pools' depending on the extent of interaction between water and macromolecules, membranes etc. NMR is typically used to determine the number of water pools based on differences in the water proton relaxation rates. In porcine meat, three different water pools were identified, the fastest relaxing reflecting water tightly associated with macromolecules, the intermediate reflecting water located within highly organized protein structures and the slowest relaxing reflecting the extra-myofibrillar water containing the sarcoplasmic protein fraction.<sup>1</sup> In fresh and prefrozen cod, respectively, two to three and three (intact, minced and centrifuged cod) and four (minced cod) different water pools have been identified, the number depending on storage conditions.<sup>2-4</sup> In the literature, water in food is typically referred to as free or bound. Although there are no clear definitions of the two states, bound water often refers to surface moisture and the first few layers of hydration, whose physical properties (relaxation times, polarisation, partial molecular volume) differ from those of bulk water.<sup>5-7</sup> Bound water constitutes the fastest relaxing pool mentioned above. The free water is bulk water and corresponds to the slowest relaxing pools determined by NMR.

Freezing is an effective way of preservation because the crystallisation of water results in a more concentrated solution and thus a lowering of the water activity in the food. Bound water or water of hydration is, however, affected by the presence of macromolecules, membranes and other ultrastructural elements in cells, tissues and foods and remains unfrozen at tens of degrees Celsius below the equilibrium freezing point of a bulk solution. A high viscosity due to the low temperature and the high concentration of protein may prevent formation of the tiny ice crystals necessary for ice formation. But even in the presence of ice crystals, some water remains unfrozen due to a combination of the hydration effect, the presence of small solutes, very high viscosity and small dimensions between membranes and macromolecules.<sup>7</sup> It is therefore frequently named 'unfreezable' water, but in compliance with the recommendations of Franks<sup>8</sup> and Wolfe *et al.*,<sup>7</sup> it is referred to as 'unfrozen water' throughout the present paper. The existence of an unfrozen water fraction in frozen foods is the main reason

why chemical quality deteriorating processes occur in frozen foods. Several experimental values for the fraction of unfrozen water in different muscle foods are reported in the literature: 9.5 % in cod muscle at -40 °C,<sup>9</sup> 11.0 % in haddock muscle at -40 °C,<sup>10</sup> 15.1 % in reindeer meat at -80 °C,<sup>11</sup> 31.0 % in fresh ground beef meat at -40 °C<sup>12</sup> and 36.7 % in king fish at -90 °C.<sup>13</sup>

The freezing point of a food is a critical factor for the determination of many physical properties such as freezing time (Planck's equation), water activity, water distribution, amount of frozen water and thawing time.<sup>14</sup> A freezing point depression of about one degree Celsius would be expected in bulk muscle water due to the presence of different salts/solutes.<sup>6,7,11</sup> But as mentioned, water can also remain unfrozen at lower temperatures due to super-cooling. Super-cooled water is in a non-equilibrium state and in the absence of ice nucleators it can remain unfrozen almost indefinitely.<sup>7</sup> The freezing point is often referred to as 'the equilibrium freezing point' but the term 'Initial freezing point' is also used and refers to the ice crystallisation temperature which is below the equilibrium freezing point due to super-cooling.<sup>14</sup> The equilibrium freezing point is equal to the melting point and is often estimated from differential scanning calorimetry (DSC) thermograms using either the inflexion point at the left part of the endothermic melting peak<sup>13</sup> or the so-called 'onset temperature' which is the intercept between the tangent at this inflexion point and the baseline. Rahman,<sup>15</sup> Kasapis *et al.*,<sup>16</sup> Sablani *et al.*<sup>17</sup> and Rahman *et al.*<sup>18</sup> also used the cooling/freezing curve method. Reported equilibrium freezing points of fish muscle and seafood are: -0.68 °C for king fish,<sup>13</sup> -1.4 °C for tuna,<sup>18</sup> -0.9 °C for abalone,<sup>17</sup> values between -0.5 and -2.1 °C for squid, calamari, scallop, cuttle, mussel, octopus, and king prawn,<sup>14</sup> -0.83, -0.91, -0.83 °C for haddock, cod and sea perch respectively<sup>19</sup> and -5 °C for tuna.<sup>20</sup> The ice crystallisation point is, however, of higher practical importance than is the equilibrium freezing point during storage of foods, if the equilibrium is never established.

In the present study, the ice crystallisation point of different fish (cod, herring, tuna) muscle was determined in a simple way as just below the lowest temperature at which no endothermic melting curve was present when heating the sample above the melting temperature in a DSC. In addition, the correlation between annealing time, temperature and the fraction of unfrozen water in the fish muscle was established. It was found that ice formation in fish muscle is an 'all possible or none' phenomenon. Either ice is not

formed at all or, when formed below the crystallisation point, the fraction of ice is virtually independent on temperature.

## MATERIALS AND METHODS

### Samples and experimental design

Yellowfin tuna (*Thunnus albacares*), cod (*Gadus morhua*) and herring (*Clupea harengus*) were used as samples in the DSC experiments.

Two yellow fin tuna (11.3 and 13.4 kg) were bought from Vagn P. Fisk (Denmark). One tuna (used in Experiment 1) was filleted, and four steaks were cut from the muscle. Samples were cut from one steak, prepared as described in Sample preparation and analysed the same day. The other steaks were vacuum packed and stored on ice for measurements on the three following days. The samples for Experiment 1 were measured in quadruple and all samples were measured in randomised order. The other tuna, used in Experiment 3, was filleted, vacuum packed (12 mbar), frozen in a blast freezer at -40 °C and stored at -40 °C for two months. After thawing, half of the samples were prepared as described in Sample preparation and measured during the day. The rest of the tuna meat was stored on ice overnight and measured the following day.

In Experiment 2, farmed cod from the Faroe Islands were frozen and stored at -40 °C until use. After thawing in running water, 12 samples from a single, randomly chosen fish were prepared as described in Sample preparation. Five samples were measured on the day of preparation and the rest of the samples were stored at 2 °C for one day. The samples were measured in randomised order to avoid influence from the storage time.

Cod (from Oresund) for Experiment 3 and 4 were obtained from a local fisherman and filleted on the day of catch. One fillet (Experiment 3) was stored on ice at 2 °C and analysed during the following three days. The other fillet was vacuum-packed (12 mbar), frozen in a blast freezer at -40 °C and stored at -40 °C for two months. The frozen samples were thawed at 2 °C and subsequently stored on ice for up to three days while the samples were analysed. Herring for Experiment 3 were stored at -30 °C before thawing and preparation for analyses. After thawing they were stored on ice for up to

three days. All measurements in Experiment 3 were made in duplicate. Frozen samples for Experiment 4 were thawed and analysed on the same day.

### Sample preparation

Small (10-25 mg) samples were cut from the fish muscle, carefully avoiding connective tissue. The samples were weighed directly into aluminium pans (Perkin Elmer DSC pans, 30 µl), sealed and stored at room temperature for at most 6 hours until measured. (Earlier experiments have shown that no measurable changes of the samples take place during this period.) Samples for Experiment 2 were prepared as described above, but as it was not possible to measure 12 samples at one day, samples for the two following days were stored at 2 °C. When these samples were taken from the cold store before measurements, they were placed in a desiccator until they reached room temperature.

### Water content

Some 50 g of the muscle from which the DSC samples were cut, was minced in a blender (Krupps Speedy Pro 720) for 3 s. Portions of approximately 2 g mince were dried at 105 °C overnight, cooled in a desiccator and the change in mass measured. The water content was determined as this mass loss divided by the original sample mass. All analyses were made in duplicate.

### DSC measurements

A differential scanning calorimeter (Perkin Elmer DSC 7) was used to determine the freezing point and the percentage of frozen water in the fish samples. The calorimeter was calibrated using a sodium chloride solution (10 % w/w and  $T_e = -21.12$  °C) for temperature calibration and indium ( $T_m = 156.60$  °C and  $\Delta H_s = 28.45$  J/g) for temperature and enthalpy calibration. An empty aluminium pan was used as reference and liquid nitrogen as coolant. The fraction of unfrozen water,  $w_u$ , in a sample, i.e. the ratio between its content of unfrozen water and total water, was calculated from the area,  $\Delta H$  (sample), of the endothermic melting curve (Fig. 1) by using the formula:

$$w_u = 1 - \frac{\Delta H(\text{sample})}{m_s \cdot w_t \cdot \Delta H_s(\text{ice})}$$

where  $m_s$  is the sample mass,  $w_t$  is the water content (mass of water divided by mass of sample), and  $\Delta H_s(\text{ice})$  is the specific enthalpy change of ice melting, set to 334 J/g.

The area of the melting curve was estimated by numerical integration after subtraction of a baseline defined from the horizontal parts of the curve.

Experiment 1: The samples were equilibrated, held at 20 °C for 1 min and then cooled from 20 °C to the annealing temperature at -10, -25, -35, -47, -60 or -80 °C, annealed for 30 min and heated to 20 °C. The scanning rate was 10 °C/min in both directions.

Experiment 2: The samples were held at 20 °C for 1 min, cooled at 10 °C/min to the annealing temperature (-20 or -60 °C), annealed from 10-240 min and heated to 20 °C at 10 °C/min.

Experiment 3 and 4: The samples were scanned in the interval between -5 and -22 °C in order to see at what temperature a portion of the water in the muscle freezes. The following procedure was followed: The samples were equilibrated at 20 °C (Experiment 3) or 1 °C (Experiment 4) for 1 min, cooled to the annealing temperature at 1 or 10 °C/min, annealed from 10 min to several hours and finally heated to 20 °C at 10 °C/min. Unfrozen samples were recognised when their thermogram lacked a melting peak. In a special case, the samples were scanned from 1 to -2 °C, held for 2 min, cooled 2 degrees, held for 2 min and so on until reaching the annealing temperature at -12 or -14 °C.

Table 1 lists the different combinations of scanning rate and annealing time and temperature in the four experiments.

## RESULTS

In Experiment 1, six annealing temperatures were chosen to see if the fraction of unfrozen water decreases with decreasing temperature. The specific enthalpy changes (per g muscle) for ice melting during the subsequent heating are listed in Table 2. Annealing for 30 min at -10 °C followed by heating to 20 °C did not result in a melting curve; thus, no ice crystallisation had occurred. With annealing temperatures between -25 and -80 °C, no significant difference in fraction of unfrozen water was found.

Experiment 2 was set up to verify the results from Experiment 1 and to check if the annealing time (20 to 240 min) had any influence on the fraction of unfrozen water at -20 or -60 °C (Table 3). No significant temperature dependence was found (*t*-test), nor

was a time dependence (linear regression) at any of the two temperatures. In conclusion, the fraction of unfrozen water in the muscle does not correlate with annealing time or temperature, but ice formation seems to be an abrupt phenomenon: when passing below the ice crystallisation point, some of the water turns into ice and a constant fraction remains unfrozen, probably in a trapped non-equilibrium state.

Table 4 shows the results of Experiment 3. All water in fresh and prefrozen cod muscle remained unfrozen down to -14 to -16 °C, slightly depending on the cooling rate. At lower temperatures, prefrozen cod had a small, but significant ( $p < 0.001$ ) decrease in the fraction of unfrozen water (18.9%) compared to fresh cod (23.7%). This difference might be due to some freeze denaturation of protein in the prefrozen muscle causing a change in the distribution of water. In prefrozen herring muscle, the ice crystallisation point was around -14 °C. Only 14.2 % of the water in these samples remained unfrozen at the lower temperatures. A similar ice crystallisation point was found in prefrozen tuna except for a single sample in which ice formation took place at -12 and -10 °C also.

In Experiment 4, different combinations of annealing temperature and time were tried in an attempt to incite ice crystallisation at temperatures higher than -14 °C under various conditions. Table 5 shows the results of the experiment. Slow cooling (1 °C/min) to -12 °C or -14 °C in steps of 2 °C with annealing for 2 min at each step during the cooling did not incite crystallisation and the cod muscle remained unfrozen after 10 min annealing at the two temperatures. Increasing the annealing time to 8 hours did not result in freezing of samples at -10 °C. At -14 °C one sample froze after two hours whereas another did not freeze even during four hours of annealing.

## DISCUSSION

The main result of the present study is that no significant dependence of temperature on the fraction of unfrozen water exists even down to -80 °C. Thus all freezable water was frozen at -20 °C or even higher. This is opposite to an earlier use of -40 °C<sup>12,21-25</sup> or -70 °C<sup>6,26</sup> as reference temperatures to distinguish free and bound water or set the limit at which all freezable water is frozen.

The average fraction of unfrozen water in cod muscle found in Experiment 2 was similar to values reported by Aktas *et al.*,<sup>21</sup> Duckworth<sup>27</sup> and Sablani *et al.*<sup>13</sup> Aktas *et*

*al.*<sup>21</sup> reported the fraction of water being unfrozen in beef at -5 and -10 °C to 49 and 44 %, respectively. Between -15 and -40 °C the average fraction of unfrozen water was at a stable level at 31 % and at -50 and -65 °C the fraction of unfrozen water was reported to 29 %. They used the same principle<sup>11</sup> for calculating the fraction of unfrozen water as in this study and concluded that the unfrozen water content reached a stable level between -15 and -40 °C, though dropping further until -50 to -65 °C. In our opinion, there is no evidence in the paper for a difference in the reported values for unfrozen water between -15 and -65 °C and therefore the results are in agreement with ours. Duckworth<sup>27</sup> used differential thermal analysis (DTA) and determined the fraction of unfrozen water to be 26 to 27 % in cod muscle. Sablani *et al.*<sup>13</sup> used a method based on  $\Delta H$ -values for ice melting as a function of water content as well as a model based on the Chen<sup>28</sup> (1986) equation and freezing curve data. They reported the unfrozen water content in king fish muscle to be 37 and 31 %, depending on the method used. Riedel<sup>9</sup> and Charm and Moody,<sup>10</sup> both using calorimetric methods, reported much lower values for the unfrozen water content: 9 % at -40 °C in cod and 11 % in lean beef and haddock muscle. Bartlett,<sup>29</sup> Chen<sup>23</sup> and Pham<sup>25</sup> calculated values between 2 and 8 % unfrozen or bound water at -40 °C in cod and between 2 and 11 % in lean beef. The calculations were based on mathematical models, and both Chen<sup>23</sup> and Pham<sup>25</sup> based the calculations on Riedel's<sup>9,30</sup> experimental values. According to Pham<sup>25</sup> the calculated differences between percentage of bound and unfrozen water differed approximately 3 %. These calculations were based on the assumption of equilibrium between frozen and unfrozen water. As mentioned earlier, unfrozen water located between membranes or macromolecules should be able to remain unfrozen even in the presence of ice crystals because solutes and low temperatures can increase the viscosity to an extent where equilibrium is unreachable during a normal time period. Therefore the amount of experimentally determined unfrozen water is usually higher than the calculated values.<sup>7</sup> Still; this does not explain the difference between our results and the older results of Riedel<sup>9</sup> and Charm and Moody.<sup>10</sup>

The high annealing times in Experiment 2 were used to ensure enough time for maximal ice crystallisation. As mentioned before, it turned out that the annealing times used in our experiments did not affect the fraction of unfrozen water. Therefore, annealing times of 10 min were used for the following DSC-measurements. These focussed on the

existence of super-cooled water in cod and tuna muscle. Rather surprisingly, the water in fresh as well as prefrozen cod muscle remained unfrozen down to lower than  $-14\text{ }^{\circ}\text{C}$ . Herring muscle water remained unfrozen down to  $-12\text{ }^{\circ}\text{C}$  and the water in tuna muscle was unfrozen at  $-10\text{ }^{\circ}\text{C}$  when a fast cooling rate ( $10\text{ }^{\circ}\text{C}/\text{min}$ ) was used. For cod and herring there seemed to be a correlation between cooling rate and degree of super-cooling; this was also observed by Rahman and Driscoll<sup>14</sup> and Rahman *et al.*<sup>18</sup> Rahman *et al.*<sup>18</sup> concluded, however, that for cooling rates at or below  $1.5\text{ }^{\circ}\text{C}/\text{min}$ , no significant differences in ice crystallisation points were observed. Moreover, their results showed only a minor degree of super-cooling in tuna. In order to assure that the observed super-cooling was not a result of a too high cooling rate, we tried to incite ice crystallisation using slow cooling rates and annealing at different temperatures during cooling to the final annealing temperature. This did not result in ice crystallisation at  $-10$  or  $-12\text{ }^{\circ}\text{C}$ , and at  $-14\text{ }^{\circ}\text{C}$  it took two hours before the water in the cod sample (approximately 15 mg) froze. This means that the cod muscle is in a super-cooled, non-equilibrium state. To our knowledge, super-cooling of (fish) muscle to  $10\text{ }^{\circ}\text{C}$  below the equilibrium freezing point is not reported elsewhere in the literature. Aktas *et al.*,<sup>21</sup> when cooling meat samples to  $-5$  or  $-10\text{ }^{\circ}\text{C}$  at  $5\text{ }^{\circ}\text{C}/\text{min}$  in a DSC followed by heating to  $35\text{ }^{\circ}\text{C}$ , reported that all samples froze thus no super-cooling was observed. This finding is in disagreement with our results, though the methods used were comparable. However, Aktas *et al.*<sup>21</sup> used minced beef meat which may contain more ‘free’ water with other thermodynamic properties than the whole fish samples used in our experiment.

When DSC-samples are cooled to, say  $-20\text{ }^{\circ}\text{C}$ , the extent of super-cooling is difficult to determine because the position of the resulting freezing exotherm depends on several factors like the cooling rate and the thermal conductivity. The melting peak obtained during a successive heating is commonly used to estimate the equilibrium freezing/melting temperature. With our approach, the heating curve is also used for indirectly measuring of the ice crystallisation point by finding a narrow temperature interval within which the melting peak disappears. Very small volumes of pure solute can super-cool tens of degrees Celsius, but biological solutions usually contain ice nucleators that initiate ice crystallisation when cooled to a few degrees Celsius below zero.<sup>31</sup> Therefore it is unusual to see super-cooling as far down as  $-10$  to  $-14\text{ }^{\circ}\text{C}$  in fish



muscle. Fast cooling rates result in a rapidly rising viscosity, which might decrease the probability of ice nucleation due to low molecular mobility. This may partly explain the difference observed between ice crystallisation temperatures for fish cooled at rates of 1 and 10 °C/min. A cod sample cooled to -10 °C was kept for 8 hours without freezing. For practical reasons, longer experimental times were not used, so the maximal time the cod can remain in this super-cooled, non-equilibrium state is not known. Also, super-cooling of fish muscle to the extent described here may differ in samples of a larger scale as the probability of nucleation increases with the volume of the sample in super cooled liquids.<sup>31</sup> Our results, however, strongly indicate that fish assumed frozen stored in domestic or supermarket freezers may not be frozen at all if it has not been previously cooled to a temperature below approximately -15 °C.

## ACKNOWLEDGEMENTS

This work was financially supported by The Danish Food Industry Agency beneath The Ministry of Food, Agriculture and Fisheries. The authors wish to thank Ulrik Cold for excellent technical assistance with the DSC-measurements.

## REFERENCES

- 1 Bertram HC, Karlsson, AH, Rasmussen M, Pedersen OD, Donstrup S and Andersen H J, Origin of multiexponential  $T_2$  relaxation in muscle myowater. *J Agric Food Chem* **49**: 3092-3100 (2001).
- 2 Jensen K N, Guldager HS and Jørgensen BM, Three-way modelling of NMR relaxation profiles from thawed cod muscle. *J Aquat Food Prod Technol* **11**: 201-214 (2002).
- 3 Andersen CM and Jørgensen BM, On the relation between water pools and water holding capacity in cod muscle. *J Aquat Food Prod Technol* **13**: 13-23 (2004).
- 4 Andersen CM and Rinnan A, Distribution of water in fresh cod. *LWT* **35**: 687-696 (2002).
- 5 Kuntz ID and Kauzmann W, Hydration of proteins and polypeptides. *Adv Protein Chem* **28**: 239-345 (1974).
- 6 Ross KD, Differential scanning calorimetry of nonfreezable water in solute-macromolecule-water systems. *J Food Sci* **43**: 1812-1815 (1978).
- 7 Wolfe J, Bryant G and Koster KL, What is 'unfreezable water', how unfreezable is it and how much is there? *Cryolett* **23**: 157-166 (2002).
- 8 Franks F, Unfrozen water - Yes - Unfreezable Water - Hardly - Bound Water - Certainly Not. *Cryolett* **7**: 207 (1986).

- 9 Riedel L, Calorimetric investigation of the freezing of fish meat. *Kaeltechnik* **8**: 374-377 (1956).
- 10 Charm SE and Moody P, Bound water in haddock muscle. *Ashrae J* 39-42 (1966).
- 11 Roos YH, Phase Transitions and Unfreezable Water Content of Carrots, Reindeer Meat and White Bread Studied using Differential Scanning Calorimetry. *J Food Sci* **51**: 684-686 (1986).
- 12 Aktas N, Tulek Y, and Gokalp HY, Determination of differences in free and bound water contents of beef muscle by DSC under various freezing combinations. *J Thermal Anal* **50**: 617-624 (1997).
- 13 Sablani SS, Rahman MS, Al-Busaidi S, Guizani N, Al-Habsi N, Al-Belushi R, and Soussi B, Thermal transitions of king fish whole muscle, fat and fat-free muscle by differential scanning calorimetry. *Thermochim Acta* **462**: 56-63 (2007).
- 14 Rahman MS and Driscoll RH, Freezing Points of Selected Seafoods (Invertebrates). *Int J Food Sci Technol* **29**: 51-61 (1994).
- 15 Rahman MS, Food Properties Handbook, CRC Press, Boca Raton, FL, pp. 87-177 (1995).
- 16 Kasapis S, Rahman MS, Guizani N, and Al-Aamri M, State diagram of temperature vs date solids obtained from the mature fruit. *J Agric Food Chem* **48**: 3779-3784 (2000).
- 17 Sablani SS, Kasapis S, Rahman MS, Al-Jabri A, and Al-Habsi N, Sorption isotherms and the state diagram for evaluating stability criteria of abalone. *Food Res Int* **37**: 915-924 (2004).
- 18 Rahman MS, Kasapis S, Guizani N and Al-Amri OS, State diagram of tuna meat: freezing curve and glass transition. *J Food Engineer* **57**: 321-326 (2003).

- 19 Fikiin KA, Ice Content Prediction Models During Food Freezing: a Survey of the Eastern European Literature. *J Food Engineer* **38**: 331-339 (1998).
- 20 Agustini TW, Suzuki T, Hagiwara T, Ishizaki S, Tanaka M and Takai R, Change of K value and water state of yellowfin tuna *Thunnus albacares* meat stored in a wide temperature range (20 °C to -84 °C). *Fisheries Sci* **67**: 306-313 (2001).
- 21 Aktas N, Tulek Y and Gokalp HY, Determination of freezable water content of beef semimembranous muscle DSC study. *J Thermal Anal* **48**: 259-266 (1997).
- 22 Boonsupthip W and Heldman DR, Prediction of frozen food properties during freezing using product composition. *J Food Sci* **72**: E254-E263 (2007).
- 23 Chen CS, Thermodynamic analysis of the freezing and thawing of foods - ice content and Mollier diagram. *J Food Sci* **50**: 1163-1166 (1985).
- 24 Chen CS, Thermodynamic analysis of the freezing and thawing of foods - enthalpy and apparent specific-heat. *J Food Sci* **50**: 1158-1162 (1985).
- 25 Pham QT, Calculation of bound water in frozen food. *J Food Sci* **52**: 210-212 (1987).
- 26 Simatos D, Faure M, Bonjour E and Couach M, Physical state of water at low-temperatures in plasma with different water contents as studied by differential thermal-analysis and differential scanning calorimetry. *Cryobiol* **12**: 202-208 (1975).
- 27 Duckworth RB, Differential thermal analysis of frozen food systems. I. The determination of unfreezable water. *J Food Technol* **6**: 317-327 (1971).
- 28 Chen CS, Effective Molecular Weight of Aqueous Solutions and Liquid Foods Calculated From the Freezing Point Depression. *J Food Sci* **51**: 1537-1539 (1986).

- 29 Bartlett LH, A thermodynamic examination of the latent heat of food. *Refrig Engineer* **47**: 377 (1944).
- 30 Riedel L, Calorimetric investigation of the meat freezing process. *Kaeltechnik* **9**: 38-40 (1957).
- 31 Wolfe J and Bryant G, Cellular cryobiology: thermodynamic and mechanical effects. *Int J Refrig* **24**: 438-450 (2001).

**Table 1. Scanning rate, annealing temperature and annealing time in the DSC-experiments.**

Exp.	Samples	Scanning rate (°C/min)	Annealing time (min)	Annealing temperature (°C)
1	Fresh tuna	10	30	-10, -25, -35, -47, -60, -80
2	Prefrozen cod	10	20, 30, 60, 120, 180, 240	-20, -60
3	Prefrozen tuna and herring, fresh and prefrozen cod	1 or 10	10 or 30	-10, -12, -14, -16, -18, -20, -22
4	Fresh and prefrozen cod	1 or 10	Up to eight hours	-5, -8, -10, -12, -14, -16, -20

**Table 2.  $\Delta H_s$  (J/g) determined from the ice melting endotherm.**

Replicate	Annealing temperature (°C)					
	-10	-25	-35	-47	-60	-80
a	0	172.6	171.8	153.8	151.8	160.8
b	0	157.1	166.3	151.0	164.3	153.7
c	0	161.9	162.9	157.6	167.4	155.1
d	0	170.9	168.5	177.0	159.5	180.1

All samples were from fresh tuna. The annealing time was 30 min and the scanning rate was 10 °C/min.

**Table 3. Unfrozen water in cod muscle after 20 to 240 min annealing at -20 and -60 °C.**

Annealing temperature (°C)	Annealing time (min)	Unfrozen water (%)
-20	20	26.9
	30	29.1
	60	25.3
	120	24.0
	180	27.7
	240	28.0
-60	20	27.3
	30	25.1
	60	24.6
	120	28.8
	180	26.6
	240	28.3

The scanning rate was 10 °C/min.



**Table 4. Percent unfrozen water in fish muscle at different annealing temperatures.**

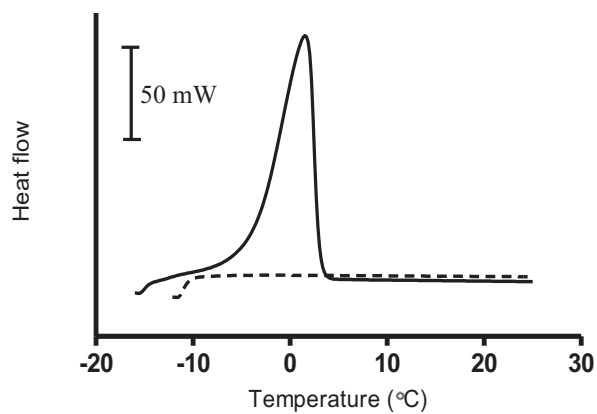
Temperature (°C)	Scanning rate (°C/min)					
	Fresh cod		Prefrozen cod		Prefrozen herring	
	1	10	1	10	1	10
-22	24.1	22.0	17.2	20.4	-	-
-20	24.1	24.6	18.4	20.5	13.1	12.7
-18	23.7	25.1	19.0	17.5	16.3	8.4
-16	23.3	100.0	19.6	100.0	16.7	15.5
-14	100.0	100.0	100.0	100.0	12.0	18.8
-12	-	-	100.0	100.0	100.0	100.0
-10	-	-		-	100.0	100.0

The annealing time was 10 min.

**Table 5. Lack of ice crystallisation in cod muscle at various combinations of annealing temperature and time.**

Annealing temperature (°C)	Annealing time (min)	Ice crystallisation
-12	2 min at respectively -2, -4, -6, -8 and -10 °C and 10 min at -12 °C	None
-14	2 min at respectively -4, -6, -8, -10 and -12 °C and 10 min at -14 °C	None
-10	60	None
-10	10	None
-10	480	None
-12	10	None
-14	10	None
-14	240	None
-14	480	Froze after 120 min

The scanning rate was 1 °C/min.



**Figure 1.** Example of DSC thermograms (heating curves) for two cod samples cooled to and annealed at -16 °C (solid line) and -12 °C (dashed line), respectively. The scanning rate was 1 °C/min. Endothermic heat flow is shown in the positive direction.